University of Alberta

Effects of Surfactants on Microbial Growth on Anthracene

by

Peng Chen

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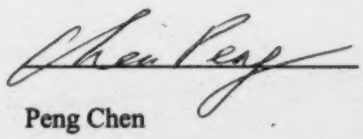

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ABSTRACT

Surfactants have been proposed as a promising method to enhance bioremediation of hydrophobic compounds in contaminated soils. However, the results of effects of surfactants on bioremediation are not consistent. This study showed that Triton X-100 at low concentration (0.024 mM or 0.09 CMC) inhibited the rate and possibly the extent of growth of either a *Mycobacterium* sp. or a *Pseudomonas* sp. on anthracene as a carbon source. The degree of inhibition of growth of the *Mycobacterium* sp. exhibited saturation when the concentration of Triton X-100 was over 0.048 mM (0.18 CMC). This study further showed that recovery of microbial growth rate could be achieved by dilution of surfactants. Further inhibition of microbial growth could be obtained by addition of more surfactants to a growing culture, but no inhibition occurred with a soluble carbon source.

This study also showed that Triton X-100 sorbed onto the surfaces of both the *Mycobacterium* sp. and the anthracene particles. Although much more surfactant sorbed onto the microorganisms (at least one magnitude higher), the fraction of surface of the *Mycobacterium* sp. covered by surfactants was of the same order of magnitude as the coverage of the anthracene surface (36% for the *Mycobacterium* sp. and 67% for the anthracene particles). Both surfaces had significant coverage. These results suggested that the surfactant altered the surfaces of the cells and the carbon source, thereby inhibiting uptake of anthracene.

The results of this work were consistent with the hypothesis that the interference with microbial adhesion to anthracene is the mechanism that underlies the inhibition of growth by Triton X-100.

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List of Abbreviations:

CMC: critical micelle concentration

HMN: 2,2,4,4,6,8,8-heptamethylnonane

NAPLs: nonaqueous phase liquids

PAHs: polycyclic aromatic hydrocarbons

PCBs: polychlorinated biphenyls

sp.: species

List of Nomenclature:

A: surface area of the PAH particles (m^2)

C_0: initial concentration of Triton X-100 (CMC)

C_1: final concentration of Triton X-100 (CMC)

C_b: bound Triton X-100 (mmol)

C_e: concentration of the free Triton X-100 (mmol)

C_{aq}: equilibrium concentration of substrate in the aqueous phase ($kg \times m^{-3}$)

C_{sat}: saturation concentration for PAH in aqueous solution ($kg \times m^{-3}$)

C_{NAPL}: concentration of substrate in the NAPL phase ($kg \times m^{-3}$)

J_{max}: maximum dissolution rate ($kg \times h^{-1}$)

k: rate constant ($m \times h^{-1}$)

k_m: saturation constant ($kg \times m^{-3}$)

K_{NAPL-W}: partition coefficient

N: amount of bound Triton X-100 (mmol)

N_c: amount of cells (cfu)

S: final amount of substrate per unit volume ($kg \times m^{-3}$)

S_0: initial amount of substrate per unit volume ($kg \times m^{-3}$)

t: time (h)

T_0: initial surface tension (dynes/cm)

T_1: final surface tension (dynes/cm)

μ: specific growth rate constant (h^{-1})

μ_{max}: maximum specific growth rate (h^{-1})

V: volume (m^3)

V_L: volume of the liquid culture (L)

X: biomass concentration ($kg \times m^{-3}$)

X_0: initial biomass concentration ($kg \times m^{-3}$)

Y: yield constant (kg biomass formed × kg substrate $used^{-1}$)

CHAPTER 1 INTRODUCTION

The contamination of soil, air, fresh water and marine environments with hazardous chemicals, for example polycyclic aromatic hydrocarbons (PAHs), is a major problem in many industrial areas. Many factors contribute to the contamination, such as accidental spills, improper storage, incineration, or the use of tar and creosote in wood-treatment (Cerniglia, 1992). Many contaminants are present as nonaqueous phase liquids (NAPLs) (Mercer and Cohen, 1990), which partition slowly into the aqueous phase and contaminate ground water (Fu and Alexander, 1995). Contaminated ground water, in turn, can pollute well water and surface water. The major groups of contaminants are: benzene, toluene, ethylbenzene and xylenes (BTEX), polycyclic aromatic hydrocarbons (PAHs), phenol, polychlorinated biphenyls (PCBs), and pentachlorophenol (PCP) (Kovalick, 1990; Lacy, 1990). Many of the above contaminants have an adverse impact on human health. For example, some PAHs such as benzo[α]pyrene are known to be carcinogens (Cerniglia, 1992). Contaminants in soil can persist for a long time and they keep releasing toxic chemicals to water supplies, therefore, they are very dangerous to human health. In this study, PAHs are the theme. Therefore, my review will focus on hydrocarbons, especially PAHs.

Several methods have been proposed to remove the contaminating compounds and reclaim these contaminated sites. They include: incineration, extraction, in-situ treatments (steam stripping, hot-air stripping), and soil washing which involves the use of water and/or surfactants. However, these methods are either very expensive or only applicable to volatile components such as benzene (Morgan and Watkinson, 1989).

Recently bioremediation has received more and more attention and is anticipated to be one of the most promising technologies for the future.

1.1 Bioremediation of Contaminated Sites

Bioremediation is a technology which involves the use of microorganisms and/or their products to degrade toxic contaminants. Depending on the target compounds, bioremediation can be done under either aerobic or anaerobic conditions (Morgan and Watkinson, 1989, Zappi et al., 1996). Generally, mixed cultures are more efficient in degrading contaminants than pure cultures (Trzesicka-Mlynarz and Ward, 1995; Preuß et al., 1997) and biodegradability of contaminants usually is inversely related to their molecular weight (Shuttleworth and Cerniglia, 1995).

Compared with the physical or chemical technologies, bioremediation has several advantages (Skinner, 1990; Baker and Herson, 1994). Biological systems are usually cheaper, potentially only 1/3 to 1/2 the cost of incineration. Remediation could be done on site which eliminates transportation costs and liabilities, and biological treatment has the potential to destroy the contaminants permanently with no harm to the environment.

However, there are still some drawbacks which hinder the wide use of bioremediation (Woodyard, 1990; Skinner, 1990). Since bioremediation is a relatively new technology, a track record of good performance has not been well established. This results in a series of uncertainties with the use of bioremediation, which include: (1) biodegradability: can the contaminants at a specific site be degraded? for example, toxic metals are not biodegradable though they may be bioaccumulated; (2) reliability: can

bioremediation obtain the high extent of destruction achieved by thermal and chemical treatment? (3) byproducts: will bioremediation produce byproducts which are also toxic or even worse than the original contaminants? (4) duration of performance: bioremediation is usually a slow process, which often takes several weeks or even several months to complete; (5) ability to treat multiple contaminants under various soil conditions.

To solve these uncertainties, more implementation of both laboratory and field-scale experimentation is required. Laboratory tests can set protocols to treat contaminated sites. Field studies can demonstrate the applicability and reliability of bioremediation for specific contaminated sites, which allows for the accumulation of a track record of the performance of bioremediation and ultimately elimination of the uncertainties of bioremediation.

1.2 Classification of Bioremediation Technologies

A classification scheme for bioremediation processes is shown in Table 1.1 (Baker and Herson, 1994). Bioremediation can be broadly divided into two main implementation categories, in-situ or ex-situ technology. In-situ technology involves the strategic introduction of additives into the contaminated zones to make remediation happen in place. This approach requires accurate knowledge of extent and location of contamination. In-situ bioremediation rate is mainly limited by the supply of additives such as oxygen (Tabak et al., 1997), therefore several physical treatments for in-situ treatments, such as venting, air stripping, and water flooding can also stimulate

Table 1.1 Bioremediation Treatment Technologies

Bioaugmentation	Addition of bacterial cultures to a contaminated medium; (Frequently used in bioreactors and ex-situ systems, but also proposed for in-situ treatment)
Biofilters	Use of microbial absorption columns to treat air emissions (in-situ and ex-situ treatment)
Biostimulation	Stimulation of indigenous microbial populations in soils and ground water (in-situ or ex-situ treatment)
Slurry Bioreactors	Biodegradation in a container or reactor; may be used to treat liquids, slurries (ex-situ treatment)
Bioventing	Stimulation of microbial growth and activity by drawing oxygen through the contaminated soil, possibly coupled with biofilters, bioaugmentation or biostimulation (in-situ treatment)
Composting	Aerobic, thermophilic treatment process which treats the mixture of contaminated material and an organic bulking agent aerobically and thermophilically; can be done using static piles, aerated piles, or continuously fed reactors (ex-situ treatment)
Landfarming	Solid-phase treatment system for contaminated soils; most commonly applied to watery sludge from refinery of crude oil (ex-situ treatment)

biodegradation by supplying oxygen to bacteria. On the other hand, ex-situ technology requires the excavation of contaminated materials and then treatment in bioreactors. This technology usually uses bioreactors. Any bioreactor process requires the establishment of a stable, active microbial population. Consequently, the growth kinetics of the degrading organisms must be established in order to allow reasonable bioreactor design.

1.3 Enhancement of Bioremediation

Many factors affect bioremediation (Cerniglia, 1992), among which the low solubility and low dissolution rate of hydrophobic compounds is generally believed to be a major limiting factor (Rouse et al., 1994). It is well known that surfactants have the ability to mobilize and solubilize hydrophobic compounds. Therefore, considerable work has been done to apply surfactants to bioremediation with the objective of increasing the supply of hydrophobic compounds to active bacteria. However, the results are not consistent (Rouse et al., 1994; Stelmack, 1997) and the mechanisms behind these results are not well understood. Another way to facilitate bioremediation is to allow microorganisms to use the hydrocarbons in NAPLs or solid hydrocarbons directly. Some microorganisms have been observed to be able to degrade solid hydrocarbons by adhering to their surfaces (Tongpim and Pickard, 1996) or degrade hydrocarbons in NAPLs by adhering to the solvent-water interface (Ortega-Calvo and Alexander, 1994).

1.4 Objectives of This Study

Previous studies have shown that surfactants inhibit microbial adhesion to surfaces and inhibited microbial growth on anthracene at low surfactant concentrations of half of the critical micelle concentration (CMC). One possible explanation for the inhibition of growth is that surfactants may interfere with the adherence of microorganisms to the target compounds (Stelmack, 1997; Stelmack et al., 1999).

The objective of this study was to use even lower concentration of surfactants to investigate their effects on the rate of microbial growth on anthracene and to determine the adsorption of surfactants to the surfaces of cells and anthracene particles. These results were interpreted in the context of Stelmack's observations on adhesion of bacteria to NAPL-water interfaces (Stelmack et al., 1999).

CHAPTER 2 LITERATURE SURVEY:

2.1 Biodegradation of Low Solubility Hydrocarbons

Many contaminants of interest for bioremediation of contaminated soils are low solubility hydrocarbon compounds, for example, PAHs. (The solubilities of some example PAHs are listed in Table 2.1 (Dzombak and Luthy, 1984)). Due to their low solubility, the microbial growth on these compounds usually exhibits different growth kinetics from the exponential growth kinetics (Prokop et al., 1971; Volkering et al., 1992; Gray et al., 1994; Lantz et al., 1995; Thibault et al., 1996).

Table 2.1 Solubilities of sample PAHs (at 25 °C)

Compound	Number of Rings	Molecular Weight	Molecular Formula	Aqueous Solubility (μg/L)
Naphthalene	2	128	$C_{10}H_8$	31700
Fluorene	3	166	$C_{13}H_{10}$	1980
Anthracene	3	178	$C_{14}H_{10}$	73
Phenanthrene	3	178	$C_{14}H_{10}$	1290
Fluoranthene	4	202	$C_{16}H_{10}$	260
Pyrene	4	202	$C_{16}H_{10}$	135
Benzo[*a*]fluorene	4	216	$C_{17}H_{12}$	45

2.1.1 Phases of Growth

The complete growth process for a batch culture can be divided into four main phases: lag phase, growth phase, stationary phase and death phase (Figure 2.1). After a culture is transferred to fresh medium, it requires a period of time to adapt to the new environment to prepare for growth; this period is called lag phase. During this phase, almost no growth can be detected. After that, cells start to reproduce at an exponential growth rate (unrestricted growth). This is the growth phase. Cells cannot keep growing indefinitely however, because some of the nutrients will be exhausted or accumulation of toxic byproducts will stop growth. The culture then enters the stationary phase where the cell division rate equals to the death rate. Finally the death rate exceeds the division rate and thereby the cultures are in death phase (Tortora et al., 1989).

2.1.2 Unrestricted Growth

If a liquid medium can provide excess nutrients for a bacterial culture to grow, then the bacterial cell grows at the maximum rate which is said to be unrestricted (Ingraham et al., 1983).

$$dX/dt = \mu X \qquad (1)$$

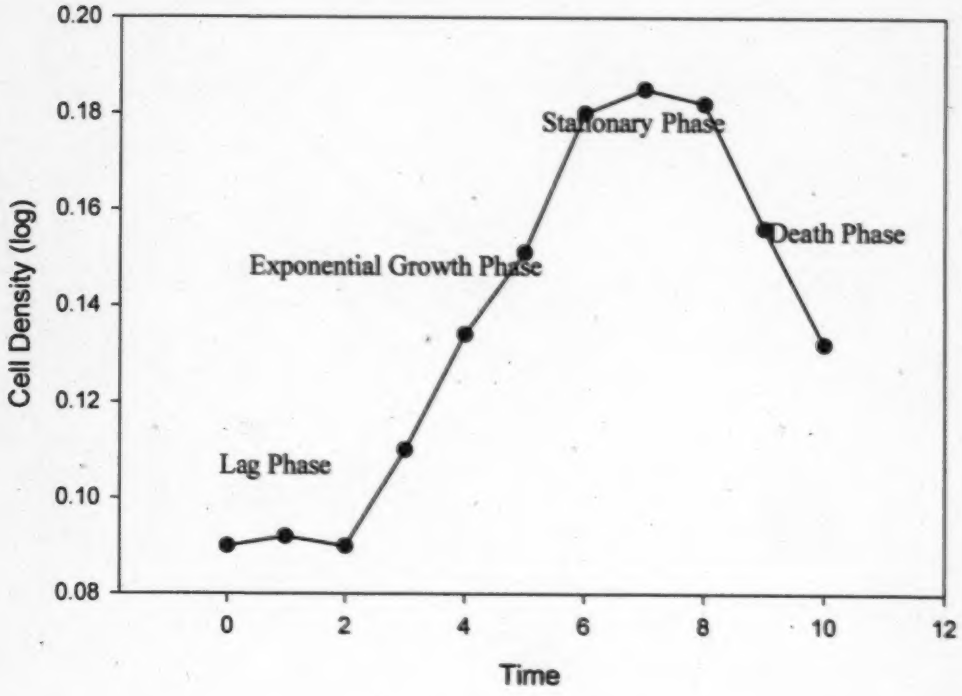
Integration of eq. 1 gives

$$X = X_0 e^{\mu t} \qquad (2)$$

According to Monod equation

$$\mu = \mu_{max} \cdot \frac{S}{S + k_m} \qquad (3)$$

Fig 2.1 Unrestricted Microbial Growth



where:

X: biomass concentration ($kg*m^{-3}$);

X_0: initial biomass concentration ($kg \times m^{-3}$) ;

t: time (h);

μ: specific growth rate constant (h^{-1}) . μ is usually used to describe how fast a particular bacterium grow in a particular environment;

μ_{max}: maximum specific growth rate (h^{-1}). μ approaches μ_{max} when the concentration of the limiting substrate increases;

k_m: saturation constant ($kg \times m^{-3}$).

From eq.2, it is clear that the culture grown under unrestricted conditions experiences exponential growth. However, for low solubility compounds, once the uptake rate by cells exceeds the dissolution rate of these compounds, the concentration of substrate starts to decrease and finally results in an insufficient supply of substrate to cells. Under these circumstances, exponential growth can not occur.

2.1.3 Biodegradation of Low Solubility Hydrocarbons

2.1.3.1 Mechanisms of Uptake of Low Solubility Hydrocarbons

The mechanisms of the uptake of low solubility hydrocarbons by microbes are still not well understood. Generally speaking, microorganisms prefer to consume dissolved hydrocarbons (Reddy et al., 1982). For example, researchers found that some pure cultures of bacteria could only use dissolved hydrocarbons (Wodzinski and Bertolini,

1972; Wodzinski and Coyle, 1974; Stucki and Alexander, 1987). This type of growth is controlled by the dissolution rate of solid hydrocarbons. In contrast, the growth rate of some microorganisms on low solubility hydrocarbon compounds was higher than the respective dissolution rate of the hydrocarbon compounds, including growth of *Candida tropicalis* on hexadecane (Yoshida and Yamane, 1971), growth of mixed cultures on octadecane (Thomas et al., 1986), and growth of *Sphingomonas sp.* on some high molecular weight PAHs (Ye et al., 1996). Explanations were proposed by these researchers. One explanation was that the microorganisms produced biosurfactants, which helped enhance the solubility of hydrocarbon compounds (Goma et al., 1974). Another possibility was that microorganisms may have the ability to degrade certain hydrophobic compounds directly without the dissolution into the aqueous phase (Zilber et al., 1980). This mechanism involves microbial adhesion to the water/hydrocarbon interface.

2.1.3.2 Types of Growth Kinetics on Low Solubility Hydrocarbons

(1) Linear Growth or Exponential Growth Followed by Linear Growth

Many previous studies showed that the growth of microorganisms on hydrocarbons is either linear or initially exponential and then linear. Examples include the growth of some yeasts on hexadecane (Prokop et al., 1971; Wang and Ochoa, 1972), growth of a *Torulopsis* sp. on n-alkanes (McLee and Davies, 1972), growth of mixed cultures of bacteria on naphthalene, 4-chlorobiphenyl and octadecane (Thomas et al., 1986), growth

of a *Flavobacterium* sp. and a *Beijerinckia sp.* on phenanthrene (Stucki and Alexander, 1987), growth of mixed cultures on soils contaminanted with lubricating oil (Rittmann and Johnson, 1989), growth of a *Pseudomonas sp.* and mixed cultures of bacteria on naphthalene, phenanthrene and anthracene (Volkering et al., 1992), growth of mixed culture on anthracene (Gray et al., 1994), growth of *Sphingomonas paucimobilis* on fluoranthene (Lantz et al., 1995), and growth of two *Pseudomonas* isolates on pyrene (Thibault et al., 1996). Some work further demonstrated that the time at which the type of growth changed from exponential to linear occurred when the hydrocarbon was no long detectable in aqueous solution (Thomas et al., 1986; Stucki and Alexander, 1987) or the concentration of dissolved hydrocarbon decreased (Boldrin, et al., 1993). These kinetics showed, therefore, a shift from unrestricted growth at early times to growth limited by the rate of dissolution of the low solubility hydrocarbon compounds (Volkering et al., 1992).

(2) Exponential Growth

Some studies showed that growth of microorganisms on low solubility organic compounds was exponential, including growth of mixed cultures on 1-naphthyl *N*-methylcarbamate (Sevin), palmitic acid and di(2-ethylhexyl) phthalate (DEHP) (Thomas et al., 1986), growth of a *Moraxella* sp. and a *Pseudomonas* sp. on biphenyl (Stucki and Alexander, 1987), and growth of a *Mycobacterium sp.* on fluoranthene (Boldrin, et al, 1993). The possible explanation is that the dissolution rates of the above low solubility organic compounds were higher than the rate of bioconversion, thereby supporting

unlimited growth. For example, Thomas et al. (1986) showed that the maximum mineralization rate of palmitic acid (29.0 ng/ml per h) was always lower than its dissolution rate (at least 70 ng/ml per h). Clearly, the observed growth kinetics depend on the balance between the dissolution of the hydrocarbon compounds and the rate of metabolism.

2.1.4 Dissolution of Low Solubility Hydrocarbons and Microbial Growth

Dissolution of low solubility hydrocarbon compounds plays an important role in determining the rate of microbial growth (Tiehm, 1994; Ye et al., 1996). The linear growth of microorganism on these compounds is often due to low solubility, which limits the supply of dissolved hydrocarbon compounds in aqueous solution.

Some researchers showed that the growth rate of microorganisms was related to the aqueous solubility of hydrocarbons and their chemical structure. The higher the aqueous solubility, the higher the growth rate (Volkering et al., 1992, Ye et al., 1996). The biodegradability of PAHs generally decreases with the increase in the number of fused benzene rings (Thakker et al, 1985; Cerniglia, 1992; Ye et al, 1996). It was also reported that with the same number of rings, the more condensed or clustered PAHs were easier to degrade than those which are less condensed (Ye et al, 1996). In general, the more benzene rings a PAH has, the lower its solubility. Consequently, the effects of molecular structure of PAHs on microbial growth is cross-correlated to the solubility of PAHs, so that the two factors are difficult to distinguish.

In contrast, some workers pointed out that water solubility was only one of the factors which governed the biodegradation rate. In that high solubility did not guarantee rapid degradation. For example, Stucki and Alexander (1987) found that a *Flavobacterium* sp. grew faster on phenanthrene than on biphenyl even though phenanthrene is less soluble in water. Obviously some other factors also have great impacts on the degradation rate, such as dissolution rate. In fact the dissolution rate is more important than solubility, because it determines the flux of energy source to the microbes. A high dissolution rate may result in a longer exponential growth phase regardless of solubility (Thomas et al., 1986; Stucki and Alexander, 1987; Boldrin et al., 1993).

Volkering et al. (1992) developed a model to describe this type of dissolution-limited linear growth kinetics. The growth rate in a batch culture is given as:

$$\frac{dX}{dt} = \frac{Y}{V} \cdot J_{\max} = \frac{Y}{V} \cdot kAC_{sat} \qquad (4)$$

where:

X: biomass concentration ($kg \times m^{-3}$);

t: time (h);

Y: yield constant (kg biomass formed × kg substrate used^{-1});

k: rate constant ($m \times h^{-1}$);

A: surface area of the PAH particles (m^2);

C_{sat}: saturation concentration for PAH in aqueous solution;

V: volume (m^3);

J_{max}: maximum dissolution rate ($kg \times h^{-1}$).

If the surface area of the PAH particles remains constant during the biodegradation process, then the above equation gives a linear growth rate which is governed by the dissolution rate of substrate.

The assumptions and limits to this equation are: (1) no cells attach to solid hydrocarbon(s); (2) there is no change in dissolution rate so that A and K are unchanged; (3) uptake rate of substrate is rapid so that the concentration of hydrocarbon(s) approaches zero in the aqueous phase ($C << C_{sat}$); (4) uptake rate of substrate is less than the maximum uptake rate for cells. The same equation would also apply to liquid hydrocarbons or hydrophobic compounds, following the same set of assumptions. In this case, the area A would be the interfacial area between the aqueous and NAPL phases.

This equation clearly shows that the microbial growth rate is directly related to dissolution rate J_{max}, which is determined by the surface area of the hydrocarbons or the interfacial area between hydrocarbons and water and the solubility of hydrocarbons. Either high dissolution rate or high solubility should result in a high microbial growth rate.

For a mixture of liquid hydrocarbons, the concentration of the biodegradable components would also affect the maximum dissolution rate. If the partition coefficient is defined as:

$$K_{NAPL-W} = \frac{C_{aq}}{C_{NAPL}} \qquad (5)$$

where:

C_{aq}: equilibrium concentration of substrate in the aqueous phase;

C_{NAPL}: concentration of substrate in the NAPL phase.

then

$$J_{max} = kAK_{NAPL-W}C_{NAPL} \qquad (6)$$

where A is the interfacial area between the NAPL and aqueous phase.

2.1.5 Enhancement of Biodegradation by Enhancing Dissolution of Low Solubility Hydrocarbons

Based on equations (4) and (6), several methods have been proposed to increase the dissolution rate, including : (1) use of small particles of hydrocarbons (Volkering et al., 1992) or small liquid droplets if hydrocarbon is in NAPL (Vogel et al., 1985); (2) use of larger amount of hydrocarbons (Keuth and Rehm, 1991); (3) agitation (Birman and Alexander, 1996). All the above three methods have been shown to be effective in experiments because they can increase the interfacial area. If attachment of cells to substrates is involved, then a larger initial inoculum may also enhance biodegradation because it may also increase the interfacial area (Ye et al., 1996).

2.1.5.1 Particle Size/Surface Area of Low Solubility Hydrocarbons

For a given shape of particle or liquid droplet, the interfacial area is inversely proportional to particle or droplet size. From equations (4) and (6), an increase in area, A, will increase dissolution rate J_{max}. Consequently, the dissolution rate is inversely related to particle size (Thomas et al., 1986; Thibault et al., 1996). The larger the particles, the

more slowly the hydrocarbon dissolves. Several studies showed that the biodegradation rate was higher with the use of smaller particles, compared with using larger particles or droplets. They include: degradation of PCBs by a *Pseudomonas sp.* (Liu, 1980); degradation of naphthalene, phenanthrene and anthracene by microorganisms (Volkering et al, 1992); degradation of pyrene by a *Mycobacterium* sp. (Boldrin et al., 1993) and degradation of fluoranthene by *Sphingomonas paucimobilis* (Lantz et al., 1995). In contrast, there are also some studies showing the bacterial growth was independent of the surface area of the solid substrate, including the biodegradation of naphthalene and bibenzyl (Wodzinski and Bertolini, 1972), and the biodegradation of phenanthrene (Wodzinski and Coyle, 1974).

Thomas et al. (1986) reported that the particle size of 4-chlorobiphenyl had no significant effects on microbial growth within the first day, however, 3 days later, it did affect the microbial growth rate. As mentioned in §2.1.3.2, the bacterial growth on 4-chlorobiphenyl exhibited biphasic kinetics. It indicates that (1) microorganisms prefer to used dissolved substrate; (2) after depletion of dissolved substrate, the microbial growth is limited by the dissolution rate of the substrate, which is directly related to the particle size.

2.1.5.2 Amount of Low Solubility Hydrocarbons

For the particles with same size, a larger amount of hydrocarbons means larger surface areas, which in turn increases the dissolution rate of hydrocarbons. Keuth and Rehm (1991) found that when they used *Arthrobacter polychromogenes* to degrade

phenanthrene, the higher the total concentration of both solid and dissolved phenanthrene, the higher the growth and degradation rates.

2.1.5.3 NAPL/Water Interfacial Area

For the degradation of hydrocarbons dissolved in NAPLs, the interfacial area between NAPLs and water is very important (Malachowsky et al., 1994). Köhler et al. (1994) reported that the biodegradation rate of phenanthrene dissolved in 2,2,4,4,6,8,8-heptamethylnonane (HMN) was mainly limited by the HMN/water interface area. Similarly, the biodegradation of hexadecane by microorganisms was enhanced with small droplet size (Fogel et al., 1985). As indicated by equation (6), any factors that increase the interfacial area will enhance dissolution and consequently enhance microbial growth.

2.1.5.4 Agitation

It is well known that agitation can help break the large solid particles or liquid droplets into smaller ones, which enhances the interfacial area. Consequently, agitation is beneficial to dissolution (Fu and Alexander, 1995) and enhances biodegradation (Fu and Alexander, 1995; Birman and Alexander, 1996).

From the above discussion, it is clear that solubility and dissolution rate of low solubility hydrocarbons are very important in bioremediation. It is well known that surfactants could enhance the apparent solubility and dissolution rate of hydrocarbon

compounds, therefore it is natural that surfactants have been proposed to facilitate bioremediation.

2.2 Surfactants

Surfactants, a contraction from the term "surface-active agents", are substances which can adsorb onto the interfaces and lower the interfacial free energies to a significant degree (Rosen, 1989). The term interface is defined as a phase boundary between any two immiscible phases, for example, between water and NAPL or water and solid hydrocarbon.

2.2.1 Structure of Surfactants

Surfactants have three characteristics: they adsorb at interfaces in an oriented fashion; they lower the interfacial tension and they form micelles (Neu, 1996). These characteristics largely result from the unique molecular structure of surfactants. The molecular structure of a surfactant is amphipathic, which consists of a group which has strong attraction for water, named the hydrophilic head and a group which has little attraction for water, named the hydrophobic tail. The hydrophobic tail causes adsorption of the surfactant molecule at the interface and enhances interaction across the interface between the adsorbed surfactant molecules and the non-aqueous phase. The hydrophilic head helps the surfactant molecules at the interface interact with the molecules of water.

The hydrophobic tail can be a halogenated or oxygenated hydrocarbon or siloxane chain, however, in most cases it is a long-chain hydrocarbon residue. The hydrophilic head is an ionic or highly polar group. According to the nature of the hydrophilic head, surfactants are classified into four types (Rosen, 1989): (1) anionic: the head of an anionic surfactant bears a negative charge; (2) cationic: the head of a cationic surfactant bears a positive charge; (3) amphoteric or zwitterionics: the head of an amphoteric surfactant bears both positive and negative charges; (4) nonionic: a nonionic surfactant bears no apparent charge, however, its head is highly polar. (Rosen, 1989)

2.2.2 Micelles and solubilization

One of the most important properties of surfactants is micelle formation. When the concentration of surfactant in solution reaches a high enough level, surfactants start to aggregate to form colloidal-sized clusters in solution. The individual colloidal-sized clusters are called micelles and that critical concentration is called the critical micelle concentration (CMC) (Rosen, 1989; Neu, 1996)

Four types of micelles have been observed: (1) spherical; (2) elongated cylindrical with hemispherical ends; (3) large, flat lamellar micelles; and (4) vesicles (Rosen, 1989). However, all types of micelles have a hydrophobic interior and a hydrophilic exterior, when water is the solvent.

It is well known that surfactants can enhance the solubilization of insoluble substances. This phenomenon directly results from micelle formation. Surfactant-enhanced solubilization can be defined as the "spontaneous dissolution of a substance by

reversible interaction with the micelles of a surfactant in a solvent to form a thermodynamically stable isotropic solution" (Rosen, 1989). It was reported that nonionic surfactants have a much higher solubilizing power (Saito and Shinoda, 1967). The solubility of a normally insoluble substance increases linearly with the concentration of surfactants when the concentration of surfactants is above the CMC (Rosen, 1989, Edwards et al., 1991, Pennell et al., 1993, Liu et al., 1995). Below the CMC, usually the solubility is almost unaffected by the presence of surfactants (Rosen, 1989). However, there are some studies showing that below the CMC, the solubilities of certain hydrophobic compounds were also enhanced. One example was the enhancement of solubility of pyrene by a nonionic surfactant Triton X-100, however, this enhancement was only approximately one half of that of surfactant concentrations above the CMC (Edwards et al., 1992). A slight enhancement of solubility of phenanthrene by a nonionic surfactant (one of a series of ethyl glucoside fatty acid esters) was also observed by Grimberg et al. (1994). The mechanisms behind these observations are still not clear. One possible explanation is that the CMC value was not accurate and the presence of other substrates may lower the CMC value (Jafvert et al., 1994).

The dissolution rate of hydrocarbons is directly proportional to the concentration of surfactant above the CMC. Below the CMC, the dissolution rate is almost independent of the concentration of surfactants (Rosen, 1989). Consequently, one would expect the addition of surfactants at concentrations below the CMC to have no effect on dissolution rate of substrate. Microbial growth would, in turn, not be affected.

2.2.3 Surface Tension and Surfactants

The interface plays an important role in many applications, including many bioprocesses. The molecules at an interface have different properties from those of a bulk fluid. One difference is that they have higher potential energy, therefore, work is required to create a new interface. The minimum work required per unit area is what is usually measured as interfacial tension. The units for interfacial tension are mN/m or dynes/cm.

When a surfactant is added into two immiscible phases, the molecules of surfactant, which have lower potential energy, replace the initial molecules at the interface. This results in a lowering of the net energy of the system, which is represented by a lower interfacial tension. In the presence of surfactant, therefore, less work is required to disperse the immiscible phase into smaller and smaller droplets, and the resulting droplets will be more stable.

2.2.4 Surfactants and Soil Washing

The objective of soil washing is to remove the contaminants, which are usually hydrophobic, from the surfaces of soil and keep the contaminants suspended in the aqueous medium. One of the properties of surfactants is their cleaning power, which is named detergency (Rosen, 1989), therefore, surfactants have been proposed to facilitate soil washing processes (Abdul et al., 1990; Jafvert and Heath, 1991; Edwards et al., 1994). Hydrophobic organic compounds, such as hydrocarbons, PCBs or tetrachloroethylene can be removed effectively by washing with a surfactant solution

(Tiehm et al., 1997). It was reported that the use of surfactants in pump-and-treat operations can increase efficiency by several orders of magnitude (Fountain et al., 1991). Surfactants can help wash the contaminants by reducing interfacial tension between two immiscible phases, so that the contaminants can be dispersed and transported more easily through the flow channels in the soil. Contaminants can also be removed from the surfaces of soil due to their dissolution in the micellar phase (Abdul et al., 1992; Edwards et al., 1994).

However, there are still some concerns about the use of surfactants. Abdul et al., (1990) suggested that the use of surfactants could cause clogging of the soil pores because surfactants could (1) hydrolyze to form flocs; (2) combine to form micelles; (3) disperse soil colloids and (4) form viscous emulsions with petroleum products. This clogging of soil pores would greatly hinder the flow through the soil matrix. The toxicity and biodegradability of surfactants should also be of concern (Abdul et al., 1990, Volkering et al., 1998). The hydrocarbons mobilized by surfactants may possibly contaminate the underlying ground-water (Vanloock et al., 1975).

Based on their cleaning capacity, environmental impact and cost-effectiveness, some criteria to select and evaluate surfactants for soil washing have been proposed. The main points include: interfacial tension minimization ability (Vigon and Rubin, 1989; Abdul et al., 1992); critical micelle concentration (Vigon and Rubin, 1989; Abdul et al., 1990); extent of soil and contaminant dispersion by the surfactants (Hurtig et al., 1988; McDermott et al., 1989; Abdul et al., 1992); extent of contaminant solubilization in the surfactant solutions (Hurtig et al., 1988; McDermott et al., 1989; Abdul et al., 1992); extent of washing of the contaminants in batch tests (Abdul et al., 1992); biodegrability

of surfactants (Hurtig et al., 1988; McDermott et al., 1989; Abdul et al., 1992) and extent of recycle or reuse of surfactants (Rouse et al., 1993). Fountain et al. (1991) compared the extraction efficiency of over 100 surfactants and concluded that solubilization ability is the most important factor, however, other factors should also be considered. Therefore, the selection of surfactant(s) in soil washing should be based on a comprehensive consideration of the above criteria and job requirements.

2.2.5 Surfactants and Biodegradation

It is well known that surfactants have the ability to mobilize and solubilize hydrophobic compounds (Rosen, 1989), and it is also generally believed that the low solubility / low dissolution rate of hydrophobic compounds is a major limiting factor for biodegradation (Rouse et al., 1994). Consequently, considerable research has been conducted on the application of surfactants to biodegradation. Except for zwitterionic surfactants, the other three types (nonionic, anionic and cationic) have been examined. The results of these studies, however, are conflicting, and a general conclusion on the benefits of surfactants for biodegradation is not possible. This literature was thoroughly reviewed by Rouse et al. (1994) and Stelmack (1997). Recently published work continues to report conflicting effects of surfactants on biodegradation [Table 2.2].

Table 2.2 Reports of Effects of Surfactants on Biodegradation of PAHs

Reference	Type of Microorganisms	Surfactant			Organic Contaminant	Effect on Biodegradation
		Name	Type	Concentration		
Zappi et al., 1996	Mixed	Tween 80	Nonionic	> CMC	PAHs	No effect
Thibault et al., 1996	Gram-negative	Witconol SN 70	Nonionic	> CMC	Pyrene	Mixed
Birman & Alexander 1996	Gram-negative	Alfonic 810-60	Nonionic	> CMC	Phenanthrene	Enhanced
Tiehm et al., 1997	Mixed	Arkopal N-300	Nonionic	>CMC	PAHs	Inhibited
		Sapogenat T-300	Nonionic	>CMC	PAHs	Enhanced
Madsen & Kristensen, 1997	Mixed		Nonionic	<CMC	Pyrene and PAHs	Enhanced
Stelmack 1997	Gram-negative	Triton X-100	Nonionic	<CMC	Anthracene	Inhibited
		Dowfax 8390	Anionic	<CMC	Anthracene	Inhibited
	Gram-positive	Triton X-100	Nonionic	<CMC	Anthracene	Inhibited
		Dowfax 8390	Anionic	<CMC	Anthracene	Inhibited

Researchers have proposed several explanations or suggestions for the enhancement of biodegradation by surfactants:

- The enhancement of biodegradation may be due to the enhancement of apparent solubility of hydrophobic organic compounds in micelles. Many researchers have reported that the addition of surfactants above the CMC enhanced biodegradation (Mueller et al., 1990; Bury and Miller, 1993; Ghosh et al., 1994; Birman and Alexander, 1996, Tiehm et al., 1997). It was also observed that degradation of PAHs by a *Mycobacterium* sp. increased with increased surfactant concentrations (Tiehm, 1994). However, van Hoof and Rogers (1992) found that micellar concentrations of nonionic surfactants inhibited biodegradation of hexachlorobenzene while low concentrations (below the CMC) of surfactants showed inconsistent results. Consequently, enhancement of solubility does not necessarily result in an enhancement of biodegradation.

  There are also many studies that showed enhancement of biodegradation by surfactants below the CMC (Aronstein and Alexander, 1992; Fu and Alexander, 1995; Madsen and Kristensen, 1997). Obviously, this effect of enhancement is totally irrelevant to enhancement of solubility.

- The enhancement of biodegradation may be due to the increase of interfacial area between NAPL and water. Robichaux and Myrick (1972) argued that since the growth is related to the interfacial area of a non-aqueous liquid phase and surfactants increase the area, surfactants should increase the rate of microbial growth. This statement is supported by Liu (1980), who found that the mineralization of PCBs by a *Pseudomonas* sp. was enhanced by an anionic

surfactant. He also observed that biodegradation mainly occurred at the PCB-water interface. Therefore, he credited the enhancement of biodegradation to the increase of interfacial area by surfactant emulsification. Breuil and Kushner (1980) observed that *Acinetobacter lwoffi* could degrade hexadecane only after it had been emulsified with Triton X-100. They also explained this observation by the enhancement of interfacial area by surfactants. However, this mechanism would not occur with solid hydrocarbons or highly viscous liquids.

Some other possible explanations are that surfactant monomers can fluidize the cell membrane to make mass transfer easier into the cells (Van Hoof and Rogers, 1992) and that surfactants prevent contaminants from recoaggulating (Köhler et al., 1994). However, these explanations are mainly suggestions or assumptions. They still need to be proven by experiments.

Laha and Luthy (1991) suggested that the following mechanisms could help explain the inhibition of biodegradation by surfactants. The inhibition might be due to one of them, a combination, or even all of the mechanisms.

(1) Toxicity of surfactants.

It is well known that some surfactants are toxic to some microorganisms. For example, cationic surfactants are toxic in the mg/l range to a number of organisms (Whitekettle, 1991; West and Harwell, 1992). The toxicity of surfactants to microorganisms may be due to its membrane-damaging ability (Cserháti et al., 1991; van Hoof and Rogers, 1992). Tiehm (1994) compared the toxicity of nonionic surfactants of the alkylethoxylate type and the alkylphenolethoxylate type and found that the toxicity of surfactants decreased with increasing

hydrophilicity. Many researchers suggested that the possible explanation of inhibition of biodegradation is the toxicity of surfactants to microorganism (Tiehm, 1994; Deschênes et al., 1995a). However, in most cases the toxicity of surfactants was not verified experimentally. Several techniques were used in experiments to check the toxicity of surfactants. One is to compare the growth of microorganisms on low solubility hydrocarbons, for example PAHs, and on soluble carbon source, for example glucose, in the presence of surfactants. If the microbial growth on the low solubility hydrocarbons is completely inhibited while its growth on glucose is not affected at all, then this surfactant is not toxic to this microorganism. The inhibition of the growth of this microorganism on PAHs is due to other mechanisms. By using this test, Liu et al. (1995) proved that Triton X-100 at a concentration above the CMC was not toxic to *Escherichia coli* and neither Triton X-100 nor Brij 30 at concentrations well above the CMC was toxic to mixed cultures. Efroymson and Alexander (1991) used another way to check the toxicity of surfactants. They compared the mineralization of both hexadecane and naphthalene by an *Arthrobacter* sp. in the presence of the same amount of Triton X-100. They observed that Triton X-100 complete inhibited the mineralization of hexadecane while enhanced that of naphthalene, therefore, the toxicity of Triton X-100 to the microorganism was excluded. Unfortunately, these simple tests are rarely applied.

(2) Toxicity of surfactant micelles.

Roch and Alexander (1995) found that their data which showed inhibition of biodegradation of biphenyl was consistent with the mechanism which states that

the micelles of Triton X-100 are harmful to cell membrane. Therefore, they suggested that inhibition of biodegradation was due to the toxicity of Triton X-100 micelles. Actually this consistency with a proposed mechanism itself is not sufficient to make the above conclusion. Liu et al. (1995) used a direct method to test the possible membrane-disrupting ability of surfactant micelles. They compared the cell structures of bacteria grown on naphthalene with Triton X-100 or Brij 30 at concentrations of 10 times the CMC or without surfactants and they observed no obvious rupture of the cell membrane or lysis of the bacteria. Therefore, they suggested that micelles were not toxic to the bacteria.

(3) Preferential metabolism of surfactants.

Tiehm et al. (1997) reported that the rapid degradation of Arkopal N-300 resulted in a lack of oxygen and consequently inhibited the biodegradation of PAHs. Deschênes et al. (1995b) showed that SDS and rhamnolipid biosurfactants produced by *Pseudomonas aeruginosa* UG2 were preferential substrates for mixed cultures, which caused the inhibition of biodegradation of PAHs.

Actually, use of surfactants as an alternate carbon source does not necessarily inhibit the biodegradation of target compounds (Providenti et al., 1993). It may have no effects on biodegradation (Thibault et al., 1996) or even enhance the biodegradation. Liu et al. (1995) observed that Brij 30 was degraded along with the degradation of naphthalene by mixed culture while it still enhanced the degradation of naphthalene. Madsen and Kristensen (1997) reported the same observation. However, they also reported that when a fast-mineralized surfactant was used, enhancement of biodegradation of target compounds was significantly

lowered. Tiehm et al. (1997), who reported that a rapid degradation of Arkopal N-300 inhibited biodegradation of PAHs, also observed that a more slowly degrading surfactant, Sapogenat T-300, enhanced the biodegradation of PAHs in the same study. Therefore, it seems the effects of surfactants as preferential substrates on biodegradation is a comprehensive result of enhancement of mobilization and competition for nutrients. It seems inhibition of biodegradation of target compounds appear to occur if the degradation of surfactants is much faster than that of target compounds.

Some surfactants have been proven not to be consumed by certain microorganism(s). Examples include: Triton X-100 could not be degraded by either *Acinetobacter lwoffi* or *Pseudomonas aeruginosa* (Breuil and Kushner, 1980); none of Tween surfactants used by Guerin and Jones (1988) could be used as a sole carbon source by a *Mycobacterium* sp.; Tergitol NP-10 could not be consumed as a sole carbon source by *Pseudomonas stutzeri* P-16 (Grimberg and Aitken, 1995); Triton X-100 could be degraded by mixed cultures (Liu et al., 1995); Witconol SN70 was not a preferential substrate for a pyrene degrader (Thibault et al., 1996); and neither Triton X-100 or Dowfax 8390 could be consumed as single carbon source by a *Pseudumonas* sp. (Stelmack, 1997).

(4) Unavailability of micellized substrate.

The micellized substrate may be unavailable to microorganisms (Volkering et al., 1995). For example, Grimberg and Aitken (1995) used the nonionic surfactant Tergitol NP-10. They found that the apparent solubility of phenenthrene was ten times larger than that in water. Therefore, under any of the concentrations of

phenanthrene that they tested, the maximum specific uptake rates should be observed. However, the anticipated result was not obtained, so they concluded that micellized phenanthrene could not readily be consumed by *Pseudomonas stutzeri* P-16. However, Liu et al. (1995) estimated that the amount of mineralized naphthalene by mixed cultures was larger than the amount that could be provided from the aqueous phase, therefore, they suggested that the naphthalene micellized in either Triton X-100 or Brij 30 was also bioavailable.

(5) Surfactant-microorganism interactions.

Due to their amphipathic structure, surfactants tend to absorb to hydrophobic surfaces. This may cause interaction between surfactant and the surfaces of hydrocarbons and microorganisms.

Aiba et al. (1969) observed that the nonionic surfactants Scourol 400 and Tween 20 prevented the adsorption of *Corynebacterium guilliermondii* to the surface of an alkane oil droplet. Efroymson and Alexander (1991) observed that Triton X-100 interfered with the adsorption of an *Arthrobacter* sp. to the HMN-water interface. Stelmack (1997) found that Triton X-100 and Dowfax 8390 interfered with the adhesion of *Mycobacterium* sp. and *Pseudomonas* sp. to tars from contaminated soil. Triton X-100 also inhibited growth on anthracene, apparently by the same mechanism.

From the above review, we find that the effects of surfactants on biodegradation are quite controversial. The same mechanisms which are used to explain the enhancement of biodegradation are also used to explain the inhibition of biodegradation. The

interactions between microorganisms and surfactants are still poorly understood, therefore, the mechanisms that underly the observed effects of surfactants on bioremediation remain to be resolved.

2.2.6 Biosurfactants and Biodegradation

Biosurfactants, which contain both hydrophobic and hydrophilic portions, can be produced by microorganisms and excreted extracellularly (van Dyke et al., 1993). Biosurfactants include fatty acids, glycerides, phospholipids, lipopeptides and antibiotics (Kanga et al., 1997).

Compared with synthetic surfactants, biosurfactants may have several advantages (Parkinson, 1985): (1) they are usually less toxic to microorganisms; (2) they are usually biodegradable, which reduces potential pollution; and (3) they can be produced from renewable substrates.

However, the effects of biosurfactants on biodegradation are also conflicting, similar to synthetic surfactants. The different effects of biosurfactants on biodegradation were reviewed by Rouse et al. (1994) and Stelmack (1997). The possible explanations for effects of biosurfactants on biodegradation are similar to those for synthetic surfactants.

2.3 Microbial Adhesion, Hydrophobicity and Bioremediation

Microbial growth on hydrophobic hydrocarbon compounds is usually limited by the dissolution rate of these compounds. As discussed in §2.1.3, some researchers found that

the growth rates of some microorganisms on hydrophobic compounds were higher than the maximum dissolution rates of these compounds. Several possible reasons were proposed to explain these observations. Microorganisms could produce their own biosurfactants, which helped dissolve and those hydrocarbon compounds and increase the dissolution rate. Microorganisms could modify their cell surface properties to increase their affinity to hydrophobic compounds (Stucki and Alexander, 1987). Microorganisms may have the ability to degrade certain hydrophobic compounds directly by adhering to their surfaces, without the requirements of dissolution of those compounds first (Stelmack, 1997). For example, Zilber et al. (1980) found a Marine *Pseudomonas* sp. could grow on both solid and dissolved *n*-tetracosane and Tongpim and Pickard (1996) found that a *Mycobacterium* sp. could grow as an attached film on solid anthracene. Since microbial adhesion is an efficient method for microorganisms to give intimate contact to hydrophobic compounds, a good understanding of microbial adhesion is important for enhancing biodegradation processes.

2.3.1 The Bacteria Cell Wall Structure

The cell surface properties play an important role in microbial adhesion and these properties are most probably dependent on the components in the bacterium cell wall (Stelmack, 1997). Bacteria can be classified into two types: Gram-positive and Gram-negative based on the Gram reaction. Their cell walls differ significantly in structure (Fig 2.2 and Figure 2.3). Both types of bacteria have a rigid layer called peptidoglycan, which is composed of two sugar derivatives and a small group of amino acids. However,

peptidoglycan composes 90 percent of the cell wall of Gram-positive bacteria while it only comprises 5 to 20 percent of the cell wall of Gram-negative bacteria (Brock, 1979). Consequently, the surface of Gram-positive bacteria is mainly composed of peptidoglycan. Most Gram-negative bacteria have a true outer-wall layer, which is composed of lipid, polysaccharide, and protein. Between the outer membrane and peptidoglycan is the periplasmic space, which separates the peptidoglycan layer from the outer membrane (Ward and Berkeley, 1980).

Acid-alcohol fastness is another stain reaction. Fig 2.3 illustrates the structure of acid-fast bacteria. This kind of bacteria, for example, *Mycobacterium* sp. contains lipid components called mycolic acids, which makes the cell surface very hydrophobic (Brock, 1979, Stelmack, 1999).

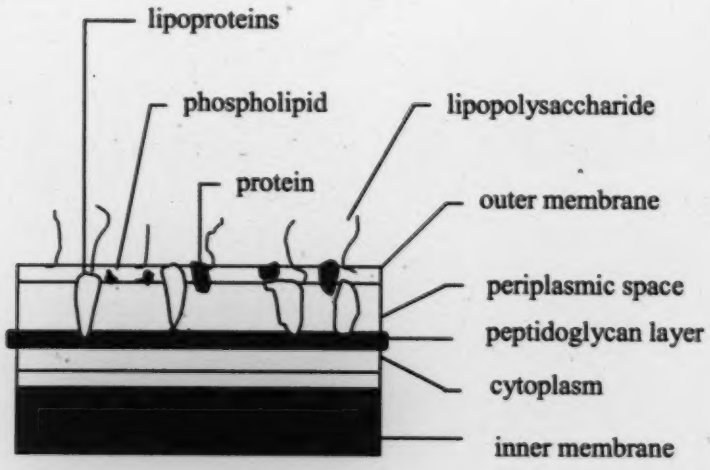


Fig 2.2 Bacterial cell wall structure - Gram-negative bacteria (Davis et al., 1980)

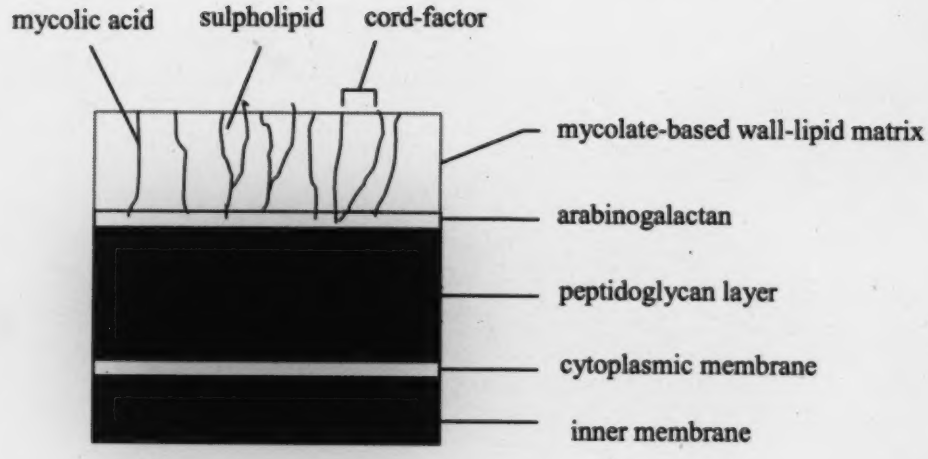


Figure 2.3 Bacterial cell wall structure - Gram-positive bacteria with mycolic acid (Minnikin and O'Donnell, 1984)

2.3.2 Microbial Adhesion and Biodegradation

Adhesion of microorganisms to the surfaces of hydrophobic compounds does not necessarily mean those microorganisms could degrade these compounds, and vice versa (Rosenberg et al., 1980, Rosenberg and Rosenberg, 1985). Examples include: *Staphylococcus aureus* and *Serratia marcescens* were found to be able to adhere to many hydrocarbons that they could not degrade (Rosenberg et al., 1980); *Acinetobacter calcoaceticus* RAG-1 adhered to hexadecane, octane and xylene with the same affinity (Rosenberg et al., 1981), however, it only degraded the first one; a *Moraxella* sp. and a

Pseudomonas sp. could degrade biphenyl while there was no presence of their cells on the surface of the solid biphenyl (Stucki and Alexander, 1987); an *Arthrobacterium* sp. could attach to the HMN/water interface regardless of the presence of substrate inside HMN (Efroymson and Alexander, 1991). These observations indicate that microbial adhesion is mainly governed by the non-specific hydrophobic interaction between microorganisms and hydrocarbons rather than the ability of bacteria to degrade the certain hydrocarbons (Reddy et al., 1982).

2.3.3 Role of Hydrophobicity in Microbial Adhesion

Several mechanisms have been proposed to explain microbial adhesion. Broadly classified, they involve either DLVO theory named after Derjaguin, Landau, Verwey and Overbeek, which emphasizes the net results of electrostatic force and van de Waals forces, or the effect of hydrophobicity (Goldstein et al., 1993). The latter mechanism is mentioned most frequently. Hydrophobicity is considered to be the most important factor, or even a determining factor in microbial adhesion (Rosenberg and Kjelleberg, 1986). Microorganisms can adhere to both hydrophobic and hydrophilic surfaces (Dahlbäck et al., 1981; Bendinger et al., 1993). However, high hydrophobicity on the cell surface favors microbial adhesion (Hogt et al., 1982; van Loosdercht et al., 1987; Bendinger et al., 1993). Bendinger et al. (1993) compared the adhesion of several strains on both hydrophobic and hydrophilic surfaces. They observed that for the same microorganism, it adhered better to hydrophobic surfaces than hydrophilic surfaces and for the same surface, the microorganisms with high hydrophobic surfaces adhered to that surface

better. This observation were also reported by Stenström (1989), and Fleminger and Shabtai (1995).

However, the mechanisms behind the adhesion due to hydrophobic interactions are still not clearly understood and quantified. The explanation of free energy reduction seems most reasonable. According to this mechanism, the water molecules around the hydrophobic surfaces are more structured than those in the bulk phase. This means that the water molecules around the hydrophobic surfaces are of higher energy. When two hydrophobic surfaces approach each other, the water molecules around these surfaces are pushed back into the bulk phase. This process, wherein a relatively ordered system changes into relatively chaotic system, results in an increase of entropy ($\Delta S > 0$), and a decrease of free energy ($\Delta G < 0$). Microbial adhesion is favored by this decrease in free energy. Surface free energy decreases with increasing hydrophobicity.

Absolom et al. (1983) suggested that the extent of adhesion is a comprehensive result of the surface properties of the microorganisms, substrate and suspending medium. For example, *Corynebacterium, Mycobacterium and Nocardium* strains contain mycolic acids, nocardols and nocardones, which make their cell surface very hydrophobic (Rosenberg and Rosenberg, 1981). These microorganisms have high affinity for hydrophobic compounds. Absolom et al. (1983) also predicted the possible relations between surface tension, which is an indication of free energy, and microbial adhesion. A better adhesion to hydrophilic surfaces should be expected when the surface tension of bacteria is larger than that of surrounding medium. When surface tension of bacteria is smaller than that of surrounding medium, better adhesion to hydrophobic surfaces should

be anticipated. The surface tensions of bacteria and solid substrate were determined by contact angle measurement in the above research.

The hydrophobicity of cell surfaces is not constant but changes during microbial growth, for example, it was reported that the older the cell, the more hydrophobic its surface (Rosenberg, 1981, Rosenberg et al., 1981; Rosenberg and Rosenberg, 1985, van Loosdrecht et al., 1990). The change is so great that sometimes it totally changes the cell surface from hydrophobic to hydrophilic or vice versa (Neu, 1996). However, Hogt et al. (1982) compared the adhesion of stationary-phase, heat-killed, formaldehye-killed and log-phase *Staphylococcus* sp. to hydrophobic FEP-Teflon films and non-hydrophobic cellulose acetate and observed no significant difference in microbial adhesion, therefore they suggested that neither growth phase nor viability of the bacteria had a significant effect on microbial adhesion. However, the microbial adhesion was strongly inhibited when the *Staphylococcus* sp. was treated with pepsin. These observations are very likely species and strain dependent.

Although hydrophobicity plays a very important role in microbial adhesion, cell surface charge and electrostatic forces may also influence microbial adhesion (Rosenberg and Rosenberg, 1981; Huysman and Verstraete, 1993). Stenström (1989) suggested that the negative charge on the cell surface had little effect on microbial adhesion while localized positive charge may affect the adhesion process, however, cell surface is normally negatively charged. It is also reported that a dynamic condition, which provided both convective and diffusive transportation of microbial particles from bulk liquid to test surfaces, favored microbial adhesion (Rijnaarts et al., 1993).

2.3.4 Effects of Surfactants on Microbial Adhesion

Surfactants have been proposed to enhance the bioavailability of hydrophobic hydrocarbon compounds. However, as reviewed in §2.2.6, the results are quite inconsistent. Surfactants have been suggested or confirmed to interact with the cell membrane, which could lead to positive or negative effects on biodegradation. One of the possible interactions is that surfactants may interfere with the microbial adhesion to a hydrophobic substrate. To check the effects of surfactants on microbial adhesion, many studies have been conducted.

Cetylpyridinium chloride (CC), a cationic surfactant, was reported to enhance the microbial adhesion to hexadecane and polystyrene (Goldberg et al., 1990). The authors suggested that the adsorption of cetylpyridinium chloride to the cell surfaces via electrostatic forces increased the hydrophobicity of cell surfaces. Sodium dodecyl sulfate (SDS), an anionic surfactant, was reported to inhibit many microbial adhesion processes, such as the adhesion of bacteria to hydroxylapatite (Nesbitt et al., 1982), the coagulation of *Actinomyces viscosus* and *Streptococcus sanguis* (McIntire et al., 1992), and the adhesion of *Thiobacillus albertis* to sulfur (Bryant et al., 1984). Triton X-100, a nonionic surfactant, inhibited the adhesion of marine bacteria to hydrophobic surfaces, but not to hydrophilic surfaces (Paul and Jeffrey, 1985). Triton X-100 was also reported to interfere with the adsorption of *Arthrobacter* sp. to the HMN-water interface (Efroymson and Alexander, 1991). Both Scourol 400 and Tween 20 were observed to prevent the adsorption of *Corynebacterium guilliermondii* to the surface of alkane oil droplets (Aiba

et al., 1969). In most of the above researches the CMC values of the surfactants used were not specified, however, the concentrations of the surfactants used were intended to be above CMC. Either Triton X-100 or Dowfax 8390, an anionic surfactant, inhibited the adhesion of both *Mycobacterium* sp. and *Pseudomonas* sp. on NAPLs and glass at their respective half CMC, however, Dowfax 8390 showed stronger inhibition. In the same study, both surfactants were effective for removing previously bound microorganisms from surfaces (Stelmack et al., 1999).

Some biosurfactants were also reported to interfere with microbial adhesion. Emulsan was reported to inhibit adherence of *Streptococcus pyogenes M-5* to buccal epithelial cells and emulsan also removed the previously bound cells (Rosenberg et al., 1983a; Rosenberg and Rosenberg, 1985). Emulsan produced by microorganisms during growth was observed to be able to significantly reduce the cell surface hydrophobicity (Huysman and Verstraete, 1993). Capsular polysaccharides were observed to reduce cell surface hydrophobicity (Bonet et al., 1993) and therefore inhibited microbial adhesion to liquid hydrocarbons (Rosenberg et al., 1983b).

The inhibition of adhesion observed in these studies may be due to the disruption of hydrophobic interaction by surfactants. For example, SDS is well known to disrupt hydrophobic interaction (Neu, 1996). It is also possible that surfactants adsorbed onto the surfaces of either cells or substrates and made the surfaces more hydrophilic (Neu, 1996).

The use of surfactants in soil bioremediation inevitably raises the question: how does the addition of surfactants alter the surface properties of cells and the hydrocarbon substrate? Microbial adhesion is believed to be an important step for microbial growth on

hydrophobic hydrocarbons in some cases, even if the adhesion is transitory (Stelmack et al., 1999) and an important mechanism to enhance the bioavailability of hydrocarbons. Previous research in this group showed that both Triton X-100 and Dowfax 8390 at a low concentration (half the CMC) inhibited growth of either a *Mycobacterium* sp. or a *Pseudomonas* sp. on anthracene. Both surfactants also inhibited the microbial adhesion to NAPLs from contaminated soils. The objective of this research was to investigate the effects of surfactants over a range of concentrations on the rate and extent of microbial growth on anthracene, and the possible relationship between these effects and microbial adhesion. Three hypotheses can be proposed here: (1) the inhibition of microbial growth on anthracene should be mitigated by lower concentrations of surfactants; (2) if the inhibition were due to interference with microbial adhesion to substrate, then the inhibition would be reversible by dilution or addition of surfactants; (3) surfactants could bind to the surfaces of either microorganisms or substrate, resulting in changes in surface properties, and consequently inhibit microbial adhesion.

In order to examine the effects of cell hydrophobicity on microbial growth on solid substrate, both a gram-positive and a gram-negative species were selected. In order to examine the effects of surfactant concentration on microbial growth on solid substrate, both a nonionic surfactant and an anionic surfactant were selected. Anthracene is a major component in contaminated soils and has a low solubility (73 μg/L). Previous work showed that a *Mycobacterium* sp. could colonize the surface of the anthracene particles (Tongpim, 1997). Therefore anthracene was chosen as the sample contaminant and sole carbon source for bacterial growth.

CHAPTER 3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Microorganisms

A gram-positive *Mycobacterium* sp. (Tongpim and Pickard, 1996) and a gram-negative *Pseudomonas* sp. (Gray et al., 1994; Stelmack et al., 1999) were used. The *Mycobacterium* sp. was identified to be close to, however, not the same as, *Mycobacterium fortuitum* based on 16S rRNA analysis. The 16S rRNA sequence data is available in the EMBO (European Molecular Biology Organization) database under accession number Y15709 (*Mycobacterium* sp. strain S1) (Stelmack et al., 1999).

The microbial growth medium consisted of 1.33 g/L KH_2PO_4, 2.67 g/L K_2HPO_4, 1 g/L NH_4Cl, 2 g/L Na_2SO_4, 2 g/L KNO_3, 0.01 g/L $FeSO_4$. $7H_2O$, and 1ml/L trace metal solution (Fedorak and Grbic'-Galic', 1991). The medium, together with anthracene at a concentration of 500 mg/L, was then autoclaved for 20 minutes at 121 °C. After autoclaving, sterile $MgSO_4.7H_2O$ was added to the medium to a concentration of 2 g/L.

3.1.2 Surfactants

Since the type of surfactant may determine the effects on microbial growth, both a nonionic (Triton X-100) and an anionic surfactant (Dowfax 8390) were used. Triton X-100, molecular weight of 625, was obtained from Rohm and Haas Company of Canada

Limited, West Hill, Ontario. Its hydrophilic head is made up of 9 to 10 polyoxyethylene units, and its hydrophobic tail is a branched 8 carbon unit (Figure 3.1). Dowfax 8390, molecular weight of 642, was obtained from Dow Chemical Company, Midland, Michigan. Its hydrophobic head is a diphenyl oxide disulfonate unit, and its hydrophilic tail is a linear 16 carbon chain (Figure 3.2).

Fig 3.1 The chemical structure of Triton X-100

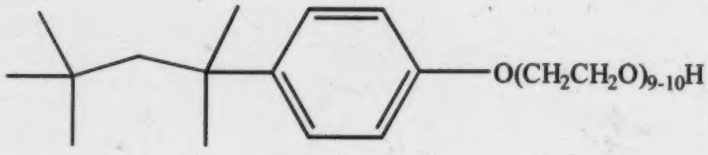
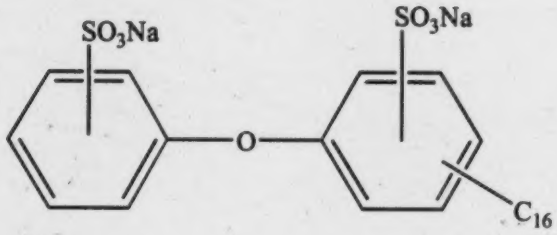


Fig 3.2 The chemical structure of Dowfax 8390



3.1.3 Other Chemicals

Anthracene, molecular weight of 178.2 and aqueous solubility of 0.070 mg/L (Cerniglia, 1992), was obtained from Sigma Chemical Company, St. Louis, Missouri, and was

reported to be 99% pure. Anhydrous D-glucose was obtained from BDH Inc., Toronto, Ontario.

3.2 Methods

3.2.1 Determination of The Critical Micelle Concentration

The part of the research was intended to determine the critical micelle concentration of both surfactants in the growth medium. A series of solution was prepared, each of which contained a different concentration of surfactant in the growth medium. The Triton X-100 concentrations ranged from 0.116 mM to 0.278 mM while the Dowfax 8390 concentrations ranged from 0.1 mM to 1.2 mM. The surface tension of each solution was measured by a surface tensiometer (Fisher Surface Tensiomat Model 21, Fisher Scientific, Hampton, New Hampshire). The surface tension was plotted against the surfactant concentration. The critical micelle concentration was determined to be the point that the surface tension no longer continued to decrease with increasing surfactant concentration (Rosen, 1989).

3.2.2 Microbial Growth on Anthracene

This part of the research was intended to determine the effects of surfactants on the microbial growth when insoluble substrate was used, and hence indirectly examine the

role of surfactants in microbial adhesion. During this part of research, the techniques and methods followed Stelmack (1997).

Microbial strains were transferred from glycerol stock at -70 °C to agar plates by streaking. The *Mycobacterium* sp. was grown on plates of plate count agar (Difco Laboratories, Detroit, Michigan) while the *Pseudomonas* species was grown on plates of trypticase soy agar (Becton Dickinson and Company, Cockeysville, Maryland). The incubation time was 7 days for the *Mycobacterium* sp. on plates of plate count agar and 3 days for the *Pseudomonas* sp. on plates of trypticase soy agar. The incubation temperature was 27 °C. After incubation, the plates were stored at 4 °C.

The microorganisms were transferred from the respective plates to 500 ml Erlenmeyer flasks. Each flask contained 100 mL growth medium and 50 mg anthracene. A 1.2 cm diameter steel coil was placed into each flask to prevent agglomeration of the microorganisms to the anthracene crystals. The flasks were incubated at 27 °C on a New Brunswick gyrotary shaker (New Brunswick, Edison, New Jersey) at 200 rpm for 14 days. The purity of both bacterial strains was checked by streaking samples on the respective plates. For the *Mycobacterium* sp., the sample was taken from each flask 3 or 4 days after being transferred from plates to medium. For the *Pseudomonas* sp., the sample was taken from each flask 6 or 7 days after being transferred from plates to medium.

For the microbial growth experiment, almost the same experimental conditions and techniques as described above were used. In this series of experiments, however, each flask contained 90 mL growth medium and was inoculated by 10 ml liquid cultures prepared with above techniques. At the end of experiment, the purity of the

Mycobacterium sp. was checked by streaking onto plate count agar and that of the *Pseudomonas* sp. was checked by streaking onto trypticase soy agar.

The optical densities of microbial solutions was measured by a UNICAM 8700 Series UV/VIS spectrometer (Unicam, Cambridge, UK) at a wavelength of 600 nm. For the *Mycobacterium* sp., an OD_{600} of 0.1 represented a cell count of approximately 10^8 cfu/ml (Tongpim and Pickard, 1996). When taking samples from each flask for OD_{600} measurement, 10-15 minutes were required for the anthracene crystals to settle down.

Cultures were grown in quadruplicate. And contaminated flasks were excluded from the data analysis. The contamination was due to the presence of other microorganism(s) which could either grow on anthracene or on surfactants. The contaminant(s) was not further examined.

3.2.3 Microbial Growth on Glucose

This part of the research was intended to investigate whether or not the surfactants were toxic to the bacteria or not. The techniques in this part of the study were only slightly different with those in §3.2.2. The differences were: (1) glucose was used as the single carbon source at a concentration of 500 mg/l; (2) it took approximately 7 days for the *Mycobacterium* sp. to consume the glucose while it took only 1 day for the *Pseudomonas* sp.

This was done in quadruplicate. However, the contaminated flasks will be excluded.

3.2.4 Reversibility of Growth Inhibition

This part of the research was designed to examine the changes in the growth of the *Mycobacterium* sp. when the concentrations of surfactants in the liquid culture were changed during its growth. The same techniques as described in §3.2.2 were employed.

This research was composed of three parallel groups of measurements. This was done in quadruplicate. However, the contaminated flasks were excluded from the data analysis.

Group 1: Initially the concentration of Triton X-100 in the liquid culture was 0.012 mM (0.05 CMC). After 4 days stock solution of Triton X-100 was added into the liquid culture to bring the concentration of Triton X-100 to 0.072 mM (0.28 CMC).

Group 2: Initially the concentration of Triton X-100 in the liquid culture (mother flasks) was 0.36 mM (1.38 CMC). After 4 days, 10 ml aliquots of liquid culture were transferred from the mother flasks to the freshly prepared growth medium (daughter flasks). This dilution gave a concentration of Triton X-100 in the daughter flasks of 0.036 mM (0.14 CMC).

Group 3: Initially the concentration of Triton X-100 in the liquid culture (mother flasks) was 0.072 mM (0.28 CMC). After 4 days, 10 ml aliquots of liquid culture were transferred from the mother flasks to the freshly prepared growth medium (daughter flasks). This dilution gave a concentration of Triton X-100 in the daughter flasks of 0.0072 mM (0.03 CMC).

3.2.5 Sorption Experiments

In order to measure the adsorption of the surfactants on the surfaces of anthracene and /or the bacteria, the following experiments were conducted.

Different amounts of solid anthracene or previously harvested bacteria were added into 125 ml Erlenmeyers flasks, each of which contained 25 ml growth medium and a known amount of Triton X-100. The control flasks contained exactly the same components as the above flasks except no anthracene was added to one series and no bacteria was added to another. The flasks were shaken at 200 rpm on a New Brunswick gyrotary shaker. After 1 day (for anthracene) or 1 hour (for the bacteria), the supernatant in the flasks was taken out and centrifuged at 18,000 rpm for 1 hour in a centrifuge (Sorvall RC-5B Refrigerated Superspeed Centrifuge, DuPont Instrument, Wilmington, Delaware), which eliminated the solid particles of anthracene or bacteria. The surface tensions of the centrifuged supernatant were measured (§3.2.1, p44). By comparing the surface tensions of the supernatant and those of the control, the Triton X-100 that was absorbed onto the surface of anthracene or bacteria could be calculated. The standard curve to relate the surface tension of medium to concentration of Triton X-100 is attached in Appendix 2.

CHAPTER 4 RESULTS AND DISCUSSION

4.1 Determination of the Critical Micelle Concentration

This series of experiments was intended to determine the CMC of both surfactants in the growth medium in order to ensure that the surfactant concentrations were below CMC.

The data of Fig 4.1 showed that as long as the Triton X-100 concentration increased from 0.12 mM to 0.26 mM, the surface tension decreased approximately linearly. However, above 0.26 mM, the surface tension remained constant with further addition of surfactant. Therefore, the CMC of Triton X-100 in the growth medium was determined to be 0.26 mM.

The data of Fig 4.2 showed that as long as the Dowfax 8390 concentration increased from 0.1 mM to 0.8 mM, the surface tension decreased. The CMC for Dowfax was determined to be 0.48 mM. Unlike Triton X-100, the CMC of Dowfax 8390 was not very distinct. This is possibly due to the fact that Dowfax 8390 is a mixture of a homologous series of surfactants (Stelmack, 1997).

Figure 4.1 Determination of the CMC of Triton X-100 in growth medium at 21 °

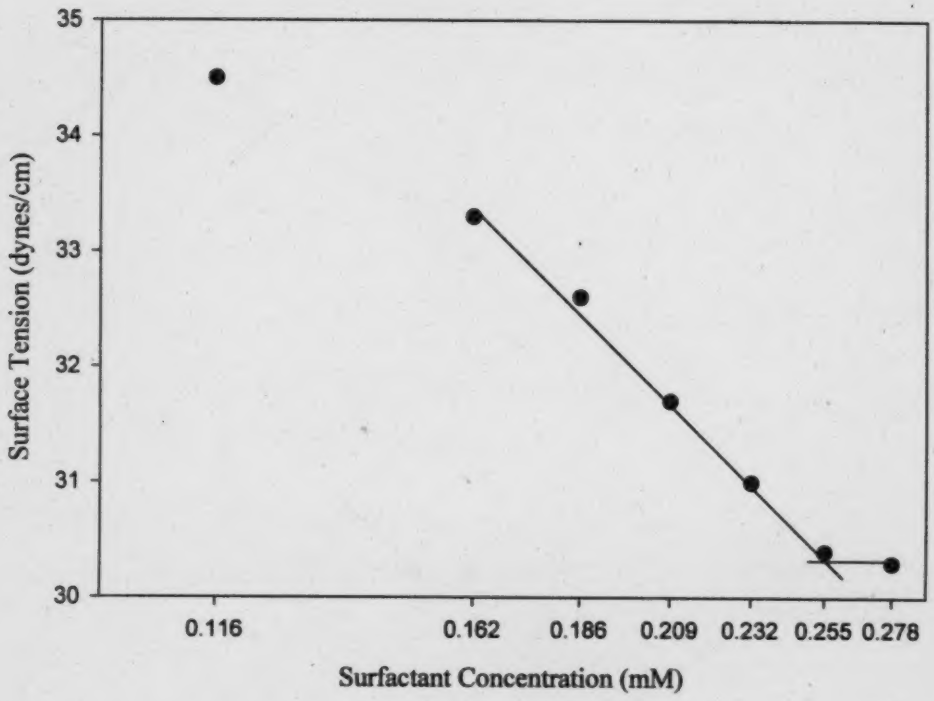
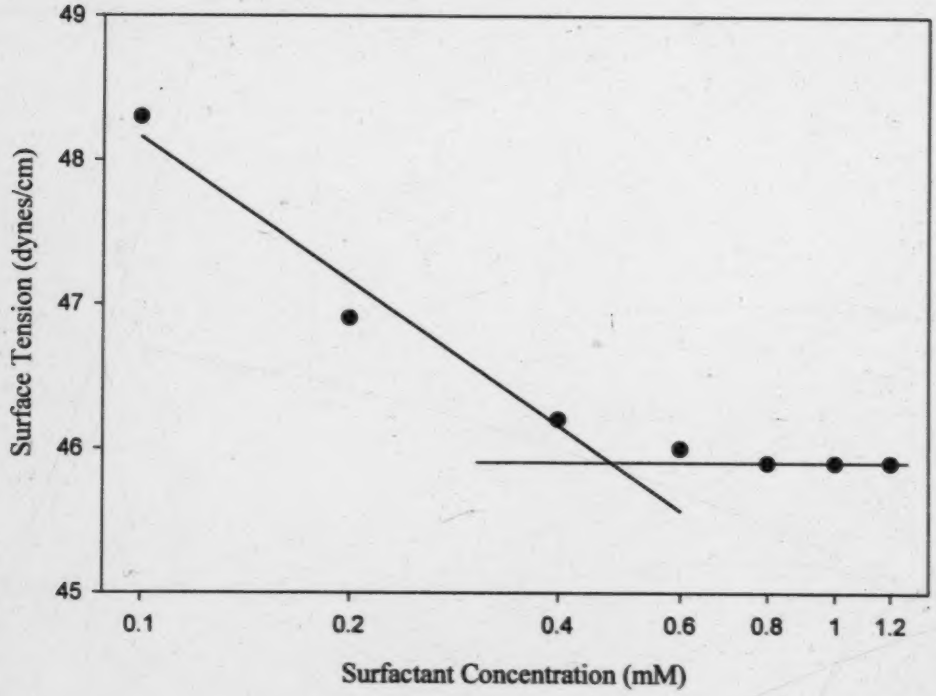


Figure 4.2 Determination of the CMC of Dowfax 8390 in growth medium at 21 ^{o}C



4.2 Microbial Growth on Anthracene

Previous research has shown that the addition of surfactants inhibited the growth rate of both the *Mycobacterium* species and the *Pseudomonas* species on anthracene (Stelmack, 1997).

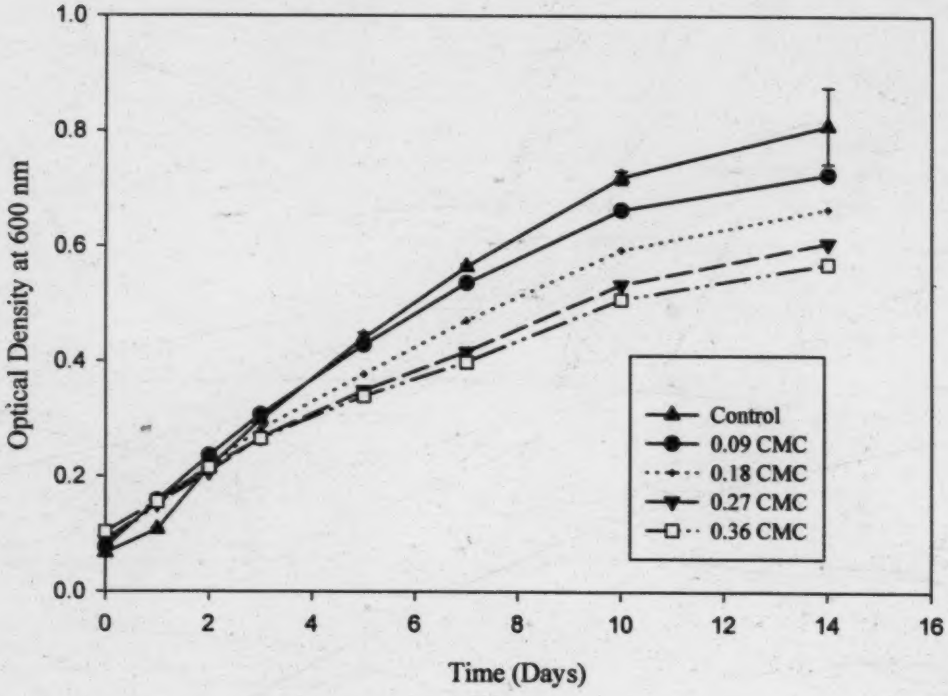
The purpose of this work is to determine the dependence of the microbial growth rate on the concentration of the surfactant. If the inhibition of microbial growth rate on anthracene resulted from interference with microbial adhesion as suggested by Stelmack (1997), then a lower concentration of surfactants will lead to less inhibition. Therefore, in this work, a range of surfactant concentrations were used.

4.2.1 *Mycobaceterium* species

Microbial growth was measured by determining OD_{600} as a function of time, as illustrated in Fig 4.3. The concentrations of Triton X-100 were from 0.024 mM to 0.096 mM (0.09 CMC to 0.36 CMC) while the concentrations of Dowfax 8390 were 0.08 mM (0.17 CMC) and 0.4 mM (0.83 CMC).

In the absence of surfactant, the *Mycobacterium* sp. growing on anthracene typically reached a maximum OD_{600} of 0.7 to 0.8 in 14 days, while the *Pseudomonas* sp. reached a density of 0.35 in 8 days. This growth behavior was comparable to the observations of Stelmack (1997) on the same strains.

Fig 4.3 Growth of *Mycobacterium* sp. on anthracene with the addition of Triton X-100 --- Series 1
(0.09 CMC to 0.36 CMC (0.024 mM to 0.096 mM); error bars of the control show the standard deviations based on four replicates)



4.2.1.1 Triton X-100

The data of Fig 4.3 showed that the presence of Triton X-100 inhibited the growth rate of the *Mycobacterium* sp. during the experimental period even when its concentration was as low as 0.09 CMC (0.012 mM). The data of Fig 4.3 showed that the OD_{600} readings of the culture without surfactant increased from about 0.07 to 0.8, which demonstrated that the culture was able to give 10-fold growth within 14 days. However, with the addition of Triton X-100, the culture grew to a much lower maximum OD_{600} reading. For example, the culture which contained Triton X-100 at 0.36 CMC (0.096 mM) could only grow from 0.07 OD_{600} to 0.4 OD_{600}. These results suggest that the presence of Triton X-100 inhibits the microbial growth rate. Three series of experiments gave the same trend and the results of repeat experiments are given in Appendix 3. Each series of experiments was done in quadruplicate. After the exclusion of contaminated flasks, the data are reported as the arithmetic average of 2 to 4 parallel experiments.

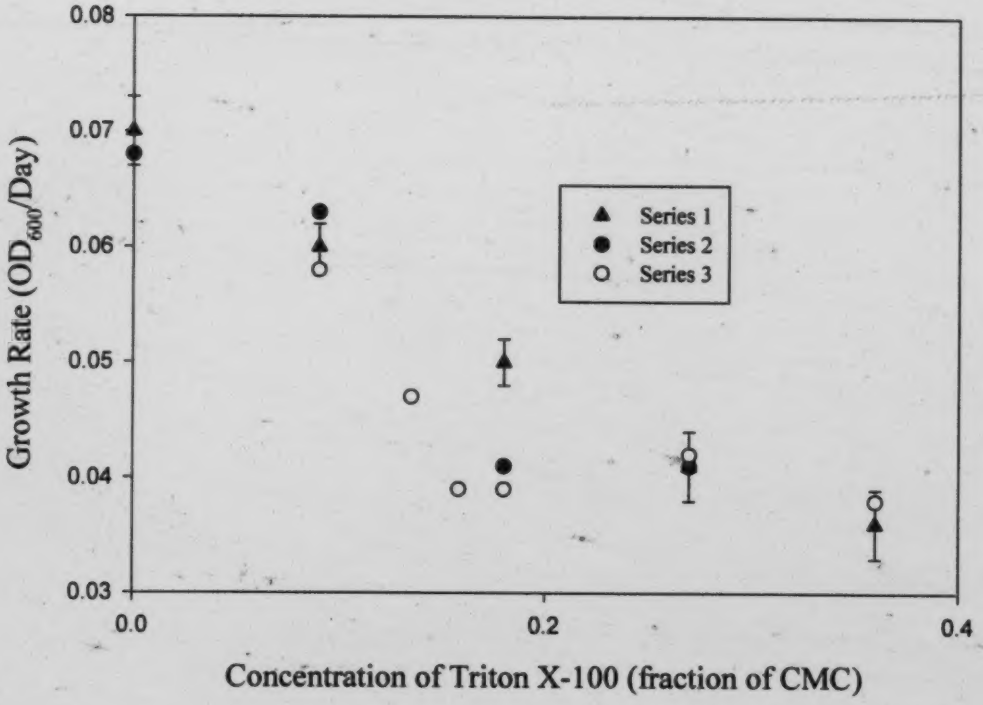
The data of Fig 4.3 showed that the bacteria started to grow at once. No apparent lag phase was observed. This observation was consistent with Stelmack's (1997) work. The growth rate of control cultures started to slow down after 7 days. Cultures growing with the presence of Triton X-100 began to slow down after 10 days, therefore, the extent of microbial growth may also be inhibited. However, since the microbial growth continued after 14 days, the inhibition of extent of microbial growth was not conclusive. During the growth period, all curves showed approximately linear growth. This result was consistent with the work of many previous researchers who observed linear growth on PAHs or low-solubility organic compounds (Prokop et al, 1971; Wang and Ochoa,

1972; Mclee and Davies, 1972; Thomas et al, 1986; Stucki and Alexander, 1987; Volkering et al, 1992; Boldrin et al, 1993, Gray et al., 1994). The likely explanation for this observation is that the low solubility of the substrate and the low dissolution rate of PAHs result in a limited supply of substrate for the bacteria. Consequently, growth was restricted by substrate supply.

The data of Fig 4.3 also showed that when more Triton X-100 was added, the bacteria grew more slowly. The data for growth from day 2 to day 7 of each concentration of Triton X-100 was used to calculate the linear growth rate. Linear regression was used in each case. The linear growth rates were then plotted as a function of the concentration of Triton X-100 in Fig 4.4. The data of Fig 4.4 clearly showed that the control, which contained no added surfactants, enjoyed the highest growth rate of around 0.070 OD_{600}/Day, while the culture which had the highest concentration of Triton X-100 (0.36 CMC / 0.096 mM) in this experiment exhibited the lowest growth rate of approximately 0.036 OD_{600}/Day. The data of Fig 4.4 also showed that when the concentration of Triton X-100 was increased from 0 to 0.18 CMC (0.048 mM), the linear growth rate dropped significantly from 0.070 OD_{600}/day to 0.043 OD_{600}/Day. However, when the concentration was further increased to 0.36 CMC (0.096 mM), the linear growth rate was only slightly changed from 0.043 OD_{600}/Day to 0.037 OD_{600}/Day. This observation suggested that the system was saturated with surfactant, so that further addition gave no further suppression of growth rate.

The data of Fig 4.4 also demonstrated good reproducibility except at 0.18 CMC (0.048 mM). Three series of experiment were conducted, and most of the data were

Fig 4.4 Growth rate of *Mycobacterium* sp. on anthracene vs. concentration of Triton X-100 (0 to 0.36 CMC (0.096 mM); error bars show the variances of data)



consistent. The error for the data at 0.18 CMC (0.048 mM) was relatively large. The likely explanation is that this point is very sensitive to small variations in surfactant concentrations. Additional information of the trend of inhibition will be provided by the data of §4.4 on the reversibility of the surfactant effects.

Since this *Mycobacterium* sp. was observed to be able to grow as an attached film on solid anthracene, it is possible that the uptake rate of anthracene by the *Mycobacterium* sp. may be higher than the maximum dissolution rate of anthracene due to the possible enhancement of consumption of solid anthracene via the close contact between cells and anthracene particles. Therefore, it is of interest for further work to determine the dissolution rate of anthracene in pure medium without microorganisms, and compare this rate with the uptake rate by the *Mycobacterium* sp.

It was possible that some cells may adhere to the anthracene particles, which would affect the OD_{600} reading. However, most of the anthracene particles settled down to the bottom of the flasks and did not go into the samples for OD_{600} reading. Also, as will be discussed in §4.6.4 (page 89), the total cell surfaces were much larger than that of the anthracene particles. For example, at day 3, the cell density was around 0.3 OD_{600} and consequently the total cell surfaces would be 0.03 m^2. If the total surface area of the anthracene particles was assumed to be the same as their initial value, 2.5×10^{-3} m^2, then the ratio of total cell surface area to that of anthracene particles would be more than 10. This number indicated that even under the condition that all the surfaces of anthracene particles were covered by cells, the free cells in liquid culture were still overwhelming. Therefore, the number of cells adhering to the anthracene particles did not significantly affect the accuracy of OD_{600} readings for cell density after 3 days while it may have some effects

within the first 3 days (the sample calculations of surface areas of cells and anthracene particles were attached in Appendix 4).

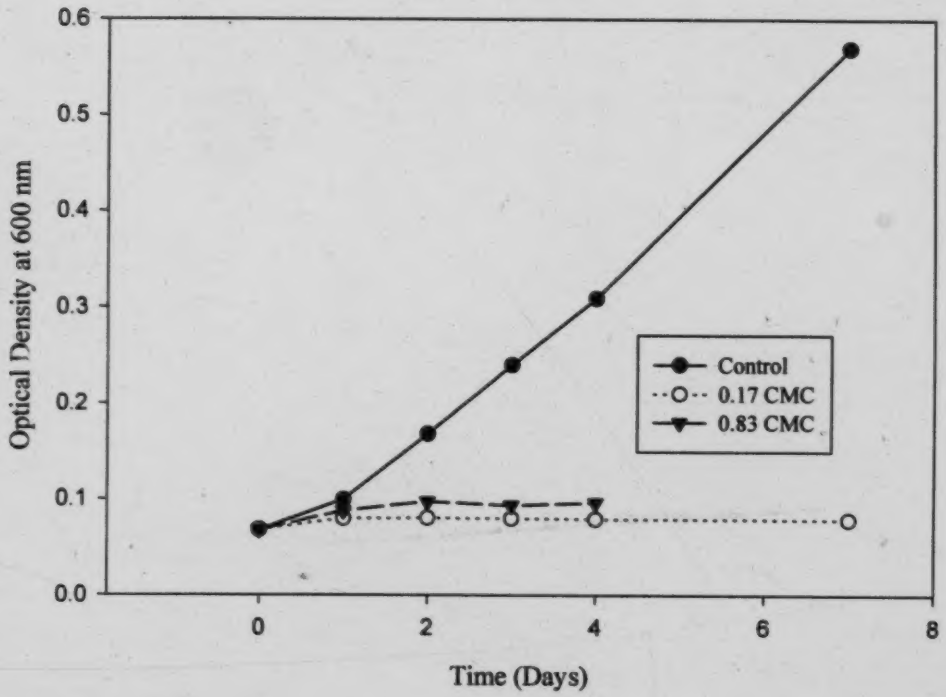
4.2.1.2 Dowfax 8390

When Dowfax 8390 was added to culture instead of Triton X-100, the *Mycobacterium* sp. showed almost no growth (Fig 4.5). The same trend was also observed by Stelmack (1997). This strong inhibition of growth may be due to the toxicity of Dowfax 8390 to the *Mycobacterium* species.

The conclusion from this series of experiments was that the presence of Triton X-100 inhibited the rate and possibly the extent of growth of the *Mycobacterium* sp. on anthracene even at concentrations of 0.09 CMC (0.024 mM). Higher concentrations of Triton X-100 gave more inhibition of growth, up to 0.18 CMC (0.048 mM). Subsequent increases in concentration gave no further suppression of growth.

Deschêne et al. (1995b) also observed that the extent of biodegradation of fluoranthene decreased with increased amount of surfactants (SDS and rhamnolipid biosurfactant). Since they only use three different concentrations of the surfactants, they did not report any saturation of inhibition. The authors also suggested that the inhibition was due to the preferential metabolism of SDS.

Fig 4.5 Growth of *Mycobacterium* sp. on anthracene with Dowfax 8390 (0.17CMC (0.08 mM) and 0.83 CMC (0.4 mM))



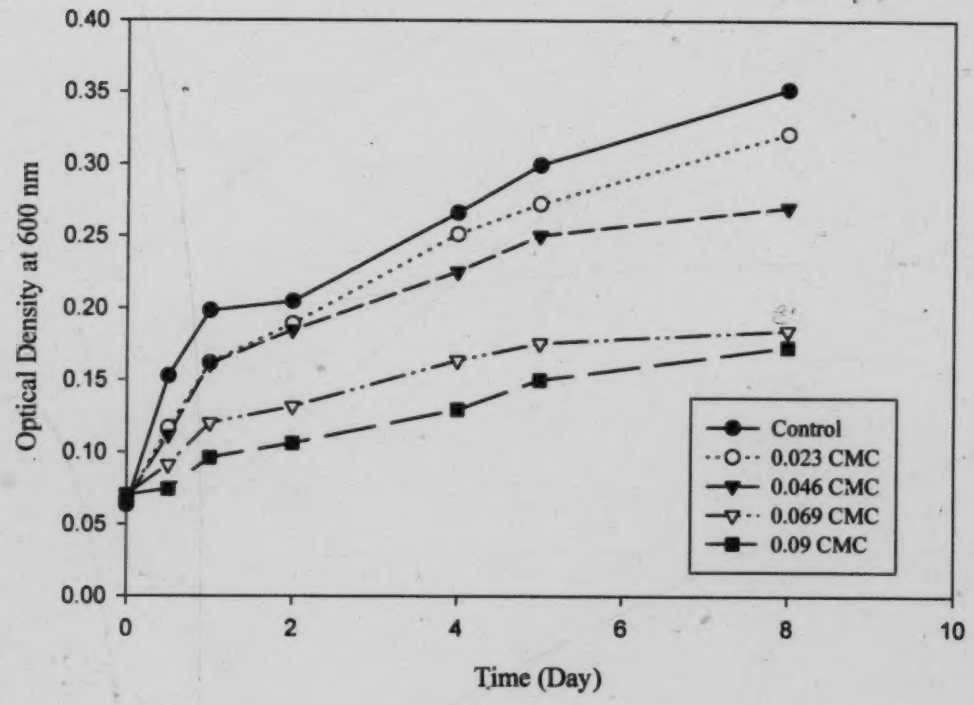
4.2.2 *Pseudomonas* species

The *Mycobacterium* sp. is Gram-positive bacterium. The experiments with Triton X-100 were repeated with a Gram-negative bacterium *Pseudomonas* sp. to determine whether the same trend of inhibition occurred. The data of Fig 4.6 clearly showed that the presence of Triton X-100 inhibited the rate and possibly the extent of microbial growth even when its concentration was as low as 0.023 CMC (0.006 mM). The control was able to grow from 0.06 to 0.35 OD_{600} while the culture with Triton X-100 at 0.09 CMC only increased from 0.06 to 0.16 OD_{600}. The data of Fig 4.6 also showed biphasic growth. During day 1, the slope of each curve was much higher than that of the section thereafter. Similar kinetics were reported previously for growth on PAHs (Thomas et al., 1986; Stucki and Alexander, 1987; Boldrin et al., 1993). The possible reason is that the growth medium after autoclaving was saturated with anthracene, therefore the initial growth was not anthracene-limited. However, once the soluble anthracene was depleted, growth was dissolution-limited. A larger initial inoculum may shorten the exponential growth phase because under this condition the soluble anthracene would be depleted faster.

This was done in quadruplicate. After the exclusion of contaminated flasks, the data was the arithmetic average of 2 to 4 parallel experiments.

The data for growth from day 1 to day 5 at each concentration of Triton X-100 was also used to calculate the linear growth rate (day 2 to day 5 for the control). These growth rates were plotted as a function of the concentration of Triton X-100 in Fig 4.7 (The sample calculation of error bars was given in Appendix 5). Fewer data were collected for the *Pseudomonas* sp., therefore, the evidence for possible saturation by

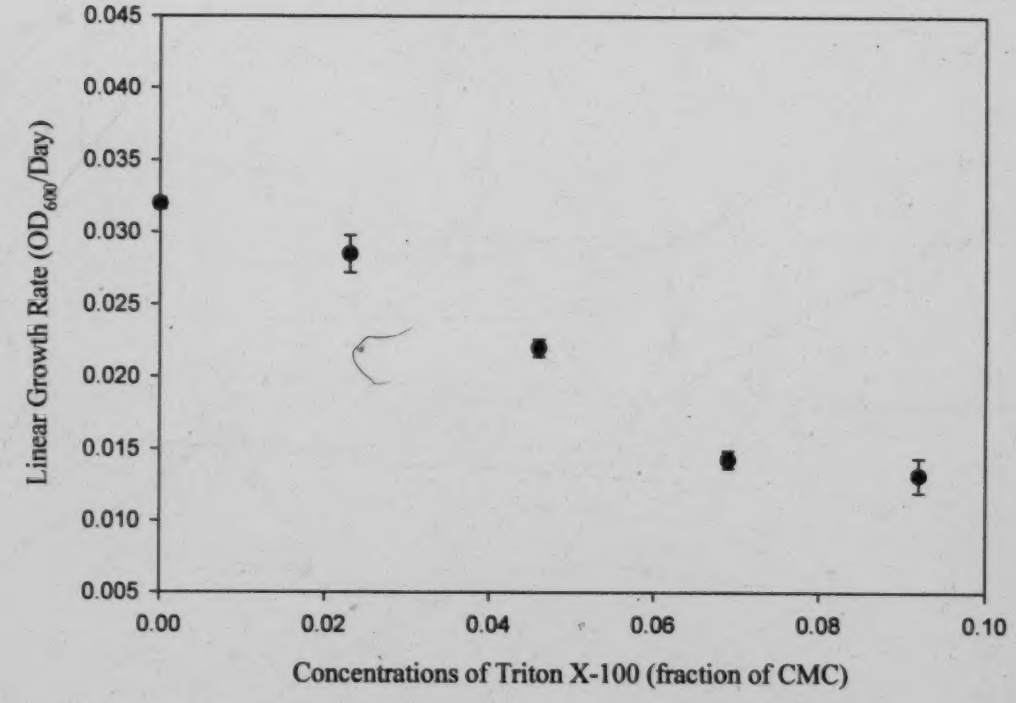
Fig 4.6 Growth of *Pseudomonas* sp. on anthracene with Triton X-100 (0.023 CMC to 0.09 CMC (0.006 mM to 0.024 mM))



surfactant was not clear. However, the data of Figure 4.6 do show significant inhibition of growth rate with increasing concentration of Triton X-100 at significantly lower concentrations than with the *Mycobacterium* sp. The maximum growth rate of the *Pseudomonas* sp. (0.032 OD_{600}/Day) on anthracene was much lower than that of the *Mycobacterium* sp. (0.070 OD_{600}/Day).

The *Pseudomonas* sp. was much more sensitive to surfactant than the *Mycobacterium* sp. At the same Triton X-100 concentration of 0.09 CMC (0.024 mM), the growth rate of the *Mycobacterium* sp. was 76% of its control while the growth rate of the *Pseudomonas* sp. was only 50% of its control. This difference in sensitivity may be related to the cell hydrophobicity. The cell wall of mycobacterial strains contain mycolic acids, nocardols, and nocardones. These components increase cell surface hydrophobicity, consequently the cell surface of *Mycobacterium* sp. is much more hydrophobic than that of *Pseudomonas* sp. (Stelmack et al., 1999). Therefore, further work is required to use different types of strains and different types of surfactants to examine the relationship between hydrophobicity and effects of surfactants on microbial growth. As proposed by Van Loosdrecht et al. (1987), the cell surface hydrophobicity could be obtained by measuring the contact angle of a drop of water on a given surface or on a closed layer of bacteria: the larger the contact angle, the more hydrophobicity the surface.

Figure 4.7 **Growth rate of *Pseudomonas* sp. on anthracene vs. concentrations of Triton X-100** (0 to 0.09 CMC (0.024 mM); error bars show the variances, from the data of Fig 4.6)



4.3 Microbial Growth on Glucose

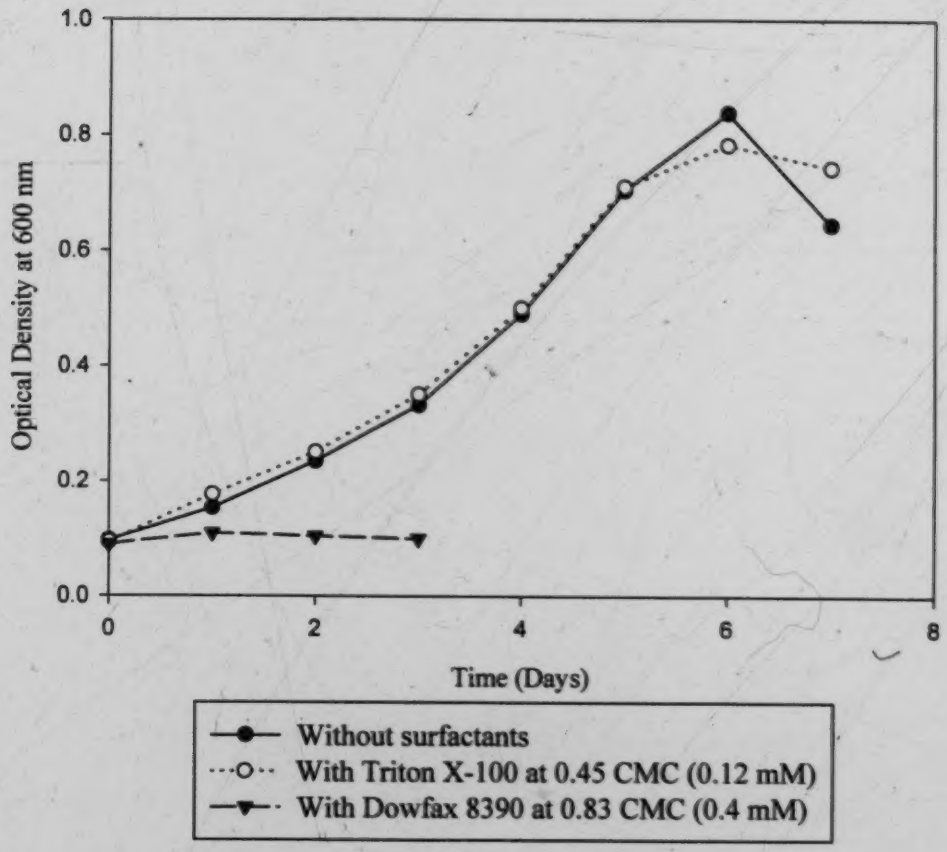
The objective of this series of experiments was to determine the toxicity of surfactants to bacteria. If the surfactants do not affect growth on glucose, then their inhibitory effect must be on uptake of anthracene. This statement is based on the assumption that normal growth on glucose indicates that cell functions are not impaired by surfactant, including metabolism of substrates, expression of enzymes and proteins and synthesis of a full range of cellular components.

4.3.1 *Mycobacterium* sp.

The data of Fig 4.8 showed that there was no significant difference between the growth curves on glucose with and without Triton X-100. In both situations, the *Mycobacterium* species exhibited almost the same growth rate. Consequently, Triton X-100 was not toxic to the *Mycobacterium* species. When Dowfax 8390 was added, the *Mycobaterium* species showed no growth (Fig 4.5). The *Mycobacterium* species consumed neither anthracene nor glucose when Dowfax 8390 was present even at low concentration (0.17 CMC or 0.08 mM for anthracene and 0.83 CMC or 0.4 mM for glucose). These results implied that Dowfax 8390 was either toxic or highly inhibitory to this Mycobacterium species.

The *Mycobacterium* sp. grew much faster on glucose (from 0.09 to 0.85 OD_{600} within7 days) than on anthracene (from 0.07 to 0.75 OD_{600} within 14 days). This observation was consistent with restricted growth on the low solubility hydrocarbon.

Fig 4.8 Growth of *Mycobacterium* sp. on glucose without or with surfactants



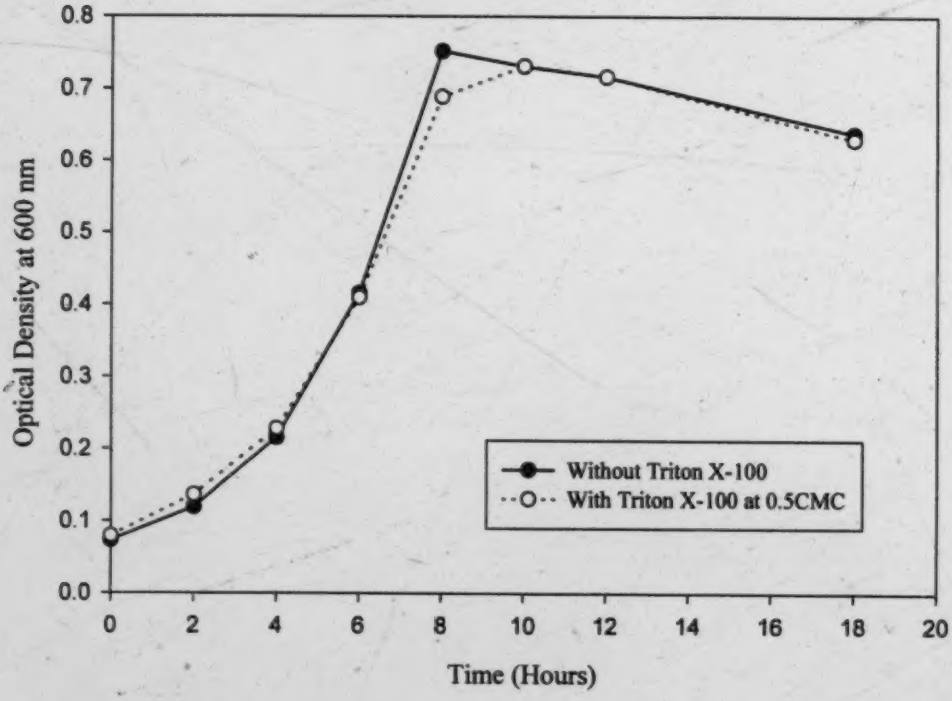
4.3.2 *Pseudomonas* sp.

Previous work showed that neither Triton X-100 nor Dowfax 8390 was toxic to the *Pseudomonas* species (Stelmack, 1997). The same experiment was repeated with Triton X-100 and the same results were observed (Fig 4.9). The lack of any inhibition of growth when glucose was the substrate showed Triton X-100 was not toxic to the *Pseudomonas* species. The lack of inhibition suggested no direct affect of surfactant on metabolism.

Note that *Pseudomonas* sp. also grew much faster on glucose (from 0.07 to 0.75 OD_{600} within one day) than that on anthracene (0.06 to 0.35 OD_{600} within 8 days).

From Fig 4.8 and Fig 4.9, we can conclude that Triton X-100 was not toxic to either the *Mycobacterium* species or the *Pseudomonas* species. Consequently, the inhibition of bacterial growth on anthracene in the presence of Triton X-100 was not due to its effect on bacterial metabolism.

Fig 4.9 Growth of *Pseudomonas* sp. on glucose without or with Triton X-100 at 0.45 CMC (0.12 mM)



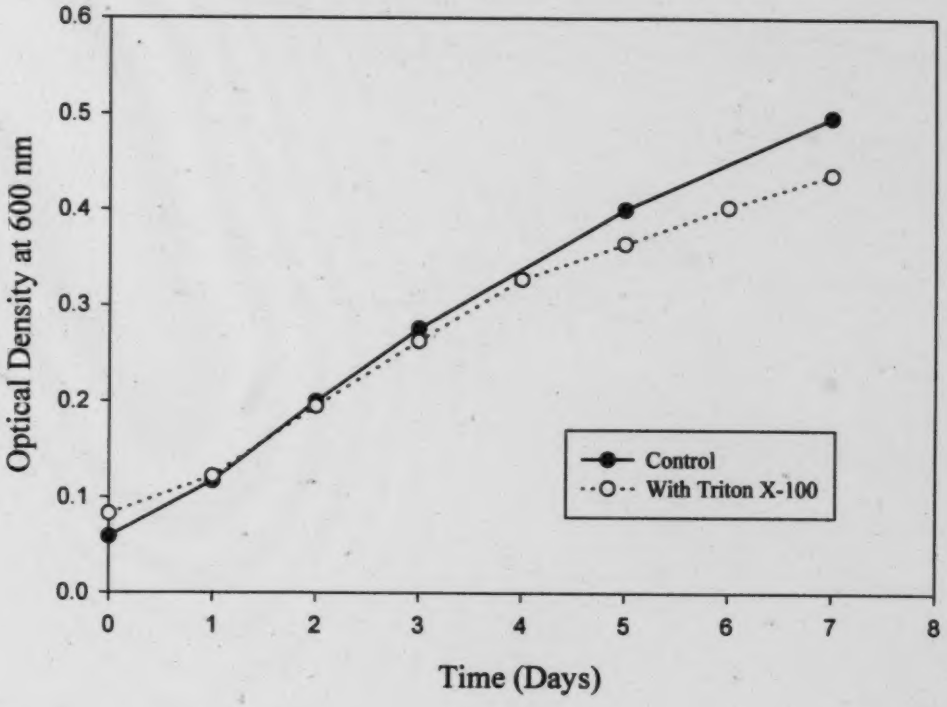
4.4 Reversibility of Surfactant Effects

The presence of Triton X-100 clearly inhibited growth on anthracene, however, the previous experiments did not show whether the surfactant had an immediate effect on growth, or whether its effects were reversible. This series of experiments was designed to answer the above questions.

4.4.1 Addition of More Triton X-100 during Microbial Growth

The data of Fig 4.10 clearly showed that when the concentration of Triton X-100 was increased from 0.05 CMC to 0.27 CMC at the end of Day 4, the growth rate of the *Mycobacterium* sp. dropped immediately. The linear growth rates before the addition of more Triton X-100 (from Day 1 to Day 4) and after the addition of more Triton X-100 (from Day 4 to Day 7) were 0.068 OD_{600}/Day and 0.037 OD_{600}/Day, respectively. The linear growth rate was decreased by almost one half. These rates were consistent with the data that was obtained in the early stage of this research (Fig 4.4). In previous experiments, the concentration of Triton X-100 was added into the medium at 0.27 CMC initially, while in this reversibility experiment Triton X-100 was added to 0.27 CMC by two steps: initially the concentration was 0.05 CMC and after 4 days the more Triton X-100 was introduced to reach a concentration of 0.27 CMC. However, the linear growth rates obtained by the above two methods were almost the same. They were 0.037 OD_{600}/Day for the two-step method and 0.041 OD_{600}/Day for the one-step method. Fig 4.10 also demonstrated that there was no obvious lag phase when the concentration of

Fig 4.10 Growth of *Mycobacterium* sp. on anthracene with the addition of Triton X-100 (concentration was changed from 0.05 CMC (0.012 mM) to 0.27 CMC (0.072 mM) at the end of Day 4)



Triton X-100 was increased from 0.05 CMC to 0.27 CMC. The bacteria adapted to the new environment very quickly and grew at the rate based on the new environment at once.

4.4.2 Dilution of Triton X-100 during Microbial Growth

Fig 4.11 represented the experiments in which the concentration of Triton X-100 was changed from 1.35 CMC (0.36 mM) to 0.14 CMC (0.036 mM). The data of Fig 4.11 showed that the addition of 1.35 CMC Triton X-100 strongly inhibited the growth of the *Mycobacterium* sp. The linear growth rate of the culture with Triton X-100 at its 1.35 CMC was only 0.016 OD_{600}/Day (linear growth rate from day 1 to day 5) while the growth rate of the control was 0.063 OD_{600}/Day (linear growth rate from day 1 to day7). These data were also consistent with the trend that was exhibited in Fig 4.4. However, the growth rate after dilution showed no obvious improvement when compared with the rate before dilution (n=3 for control; n=2 for samples with Triton X-100).

Fig 4.12 represented the experiments in which the concentration of Triton X-100 was changed from 0.27 CMC (0.072 mM) to 0.03 CMC (0.0072 mM). The data of Fig 4.12 showed that when the concentration of Triton X-100 was changed from 0.27 CMC to 0.03 CMC at the end of Day 5, the growth rate of the *Mycobacterium* sp. increased from 0.034 OD_{600}/Day (the linear growth rate from day 1 to day 8 of the mother flasks) to

Fig 4.11 Growth of *Mycobacterium* sp. on anthracene with the addition of Triton X-100 (concentration of Triton X-100 was diluted from 1.35 CMC (0.36 mM) to 0.14 CMC (0.036 mM) at the end of Day 4)

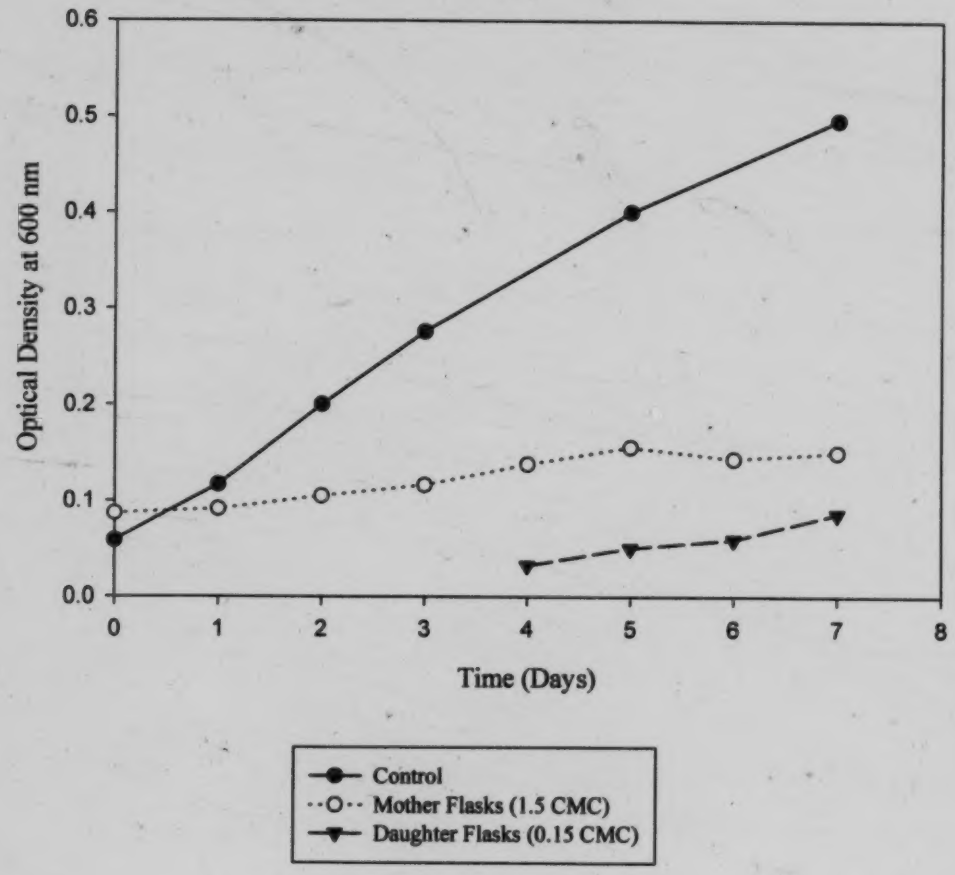
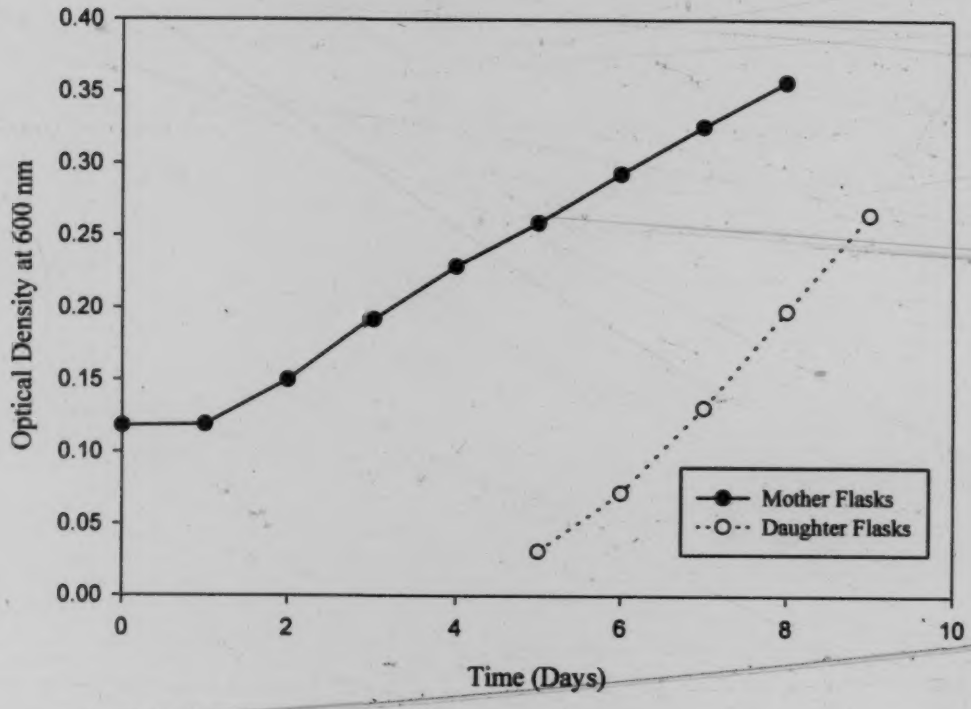


Fig 4.12 Growth of the *Mycobacterium* sp. on anthracene with the addition of Triton X-100. (The concentration of Triton X-100 was diluted from 0.27 CMC (0.072 mM) to 0.03 CMC (0.0072 mM) at the end of day 5)



0.065 OD_{600}/Day (linear growth rate from day 6 to day 9 of the daughter flasks). These rates were also consistent with the data obtained at a constant initial concentration (Fig 4.4). The data of Fig 4.12 may indicate a lag phase when the concentration of Triton X-100 was diluted from 0.27 CMC to 0.03 CMC, but this lag phase was less than one day and was probably not statistically significant (n=3 for diluted and undiluted sets of samples).

The above data suggested that the inhibition by surfactant below CMC could be recovered rapidly by dilution. For the experiments with surfactant concentration above CMC, in the experimental period (3 days), no obvious improvement in growth by dilution was obtained. However, one week later, the color in the daughter flasks was much more yellow than the color in the mother flasks. Color is an important indicator of microbial growth. Therefore, it is reasonable to suggest that the cultures in the daughter flasks eventually gave a higher growth rate than those in the mother flasks after dilution. A possible reason for the slow response was that the initial inoculum density (a OD_{600} of 0.162) after dilution is too low, which is only 1/4 of the usual inoculum density of previous study. A low initial inoculum density usually prolongs the lag phase. Some studies showed that biodegradation was insensitive to the initial inoculum (Churchill et al., 1995; Joshi and Lee, 1996). However, in those studies, the differences of initial inoculum tested were not very large, at most two-fold larger. Gray et al. (1994) observed that a ten-fold larger inoculum did affect biodegradation. Another possible reason is that the microorganism needs some time to adjust to the new environment after exposure to a high concentration of surfactant. Therefore, it is of interest for further work to examine the dependence of both the extent and possible lag phase of recovery by dilution on the

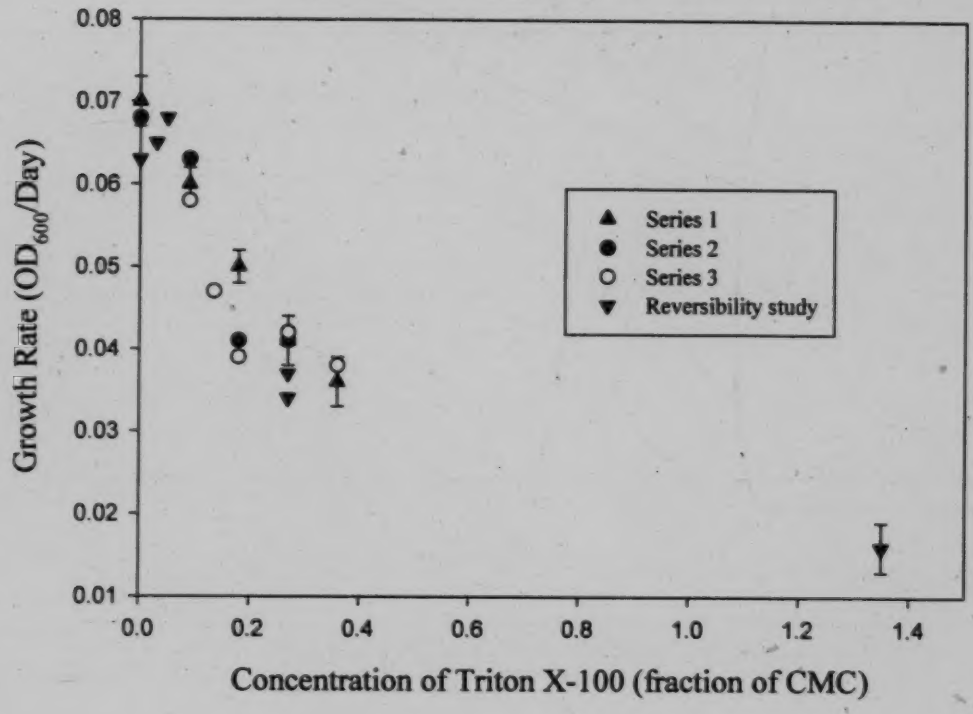
initial surfactant concentration. This can be done by the following techniques: step 1, to add different concentrations of surfactant into liquid cultures; step 2, to let the cultures grow for a period of time; step3, to take the same amount of cultures from the flasks containing different concentration of surfactants (mother flasks) and use those cultures as initial inoculum to inoculate daughter flasks; step 4, to measure the growth rates in the daughter flasks; and step 5, to correlate the recovery of growth with the initial concentration of surfactants in the mother flasks and probably with the concentration of surfactants in the daughter flasks. Note the initial inoculum for the daughter flasks should be high enough to avoid any lag phase due to low initial inoculum.

We can conclude from Fig 4.10 - 4.12 that the inhibition of microbial growth by Triton X-100 was enhanced or alleviated by introducing more Triton X-100 or diluting the existing mixture accordingly. Laha and Luthy (1991) reported that the inhibition of phenanthrene mineralization by nonionic surfactants Triton X-100 was recovered by dilution of surfactant. They found that when the concentration of surfactants was diluted from 7 CMC to 5 CMC, the mineralization of phenanthrene by mixed cultures was enhanced immediately. Laha and Luthy (1992) also reported that inhibition of phenanthrene mineralization by Triton X-100 could be mitigated by dilution of surfactant from supra-CMC to sub-CMC. In both cases, the authors suggested that the possible explanation for the above observation was that the inhibition was caused by a kind of reversible interaction between bacteria and surfactant. However, they did not further prove their hypothesis and they also did not examine the effect of addition of more surfactants.

The data for linear growth rates obtained in the reversibility experiments were added into Fig 4.4 to get Fig 4.13. The comprehensive data of Fig 4.13 demonstrated that the growth of the *Mycobacterium* sp. was inhibited by Triton X-100 at a broad range of concentration up to 1.35 CMC and likely beyond. The growth rate of the culture with addition of Triton X-100 at 1.35 CMC was 0.016 OD_{600}/Day which was only one-fourth of that of control (0.07 OD_{600}/Day).

The data of Fig 4.13 also showed that when the concentration of Triton X-100 was increased from 0 to 0.18 CMC (0.048 mM), the microbial growth rate dropped around 36% while the addition of much more Triton X-100 (6.5 times) only made the microbial growth rate dropped another 40% (based on the growth rate of control). Based on the data of Fig 4.13, two possible models can be proposed. Model 1 proposes that the inhibition of microbial growth on anthracene increased with the increased surfactant concentration, however, this inhibition reached saturation at the surfactant concentration of 0.18 CMC (0.048 mM) and further addition of surfactant gives no significant stronger inhibition. Model 2 proposes that the inhibition reached saturation at the surfactant concentration of 0.18 CMC and this saturation continues to up to 1 CMC. After the CMC, more surfactant led to stronger inhibition again.

Fig 4.13 Growth rate of *Mycobacterium* sp. on anthracene vs. concentration of Triton X-100 (0 to 1.35 CMC (0.096 mM); error bars show the variances of data)



4.5 Sorption of Surfactants on Surfaces

Previous research has shown that surfactants could interfere with microbial adhesion to hydrocarbon and NAPL surfaces (Aiba et al., 1969; Efroymson and Alexander, 1991, Stelmack et al., 1999). This kind of interference may result in inhibition of biodegradation. One possible mechanism for this interference is that surfactants may adsorb onto the surfaces of either substrate or microorganism or even both (Neu, 1996). This part of the research was to investigate how surfactants partition between these surfaces and the aqueous phase.

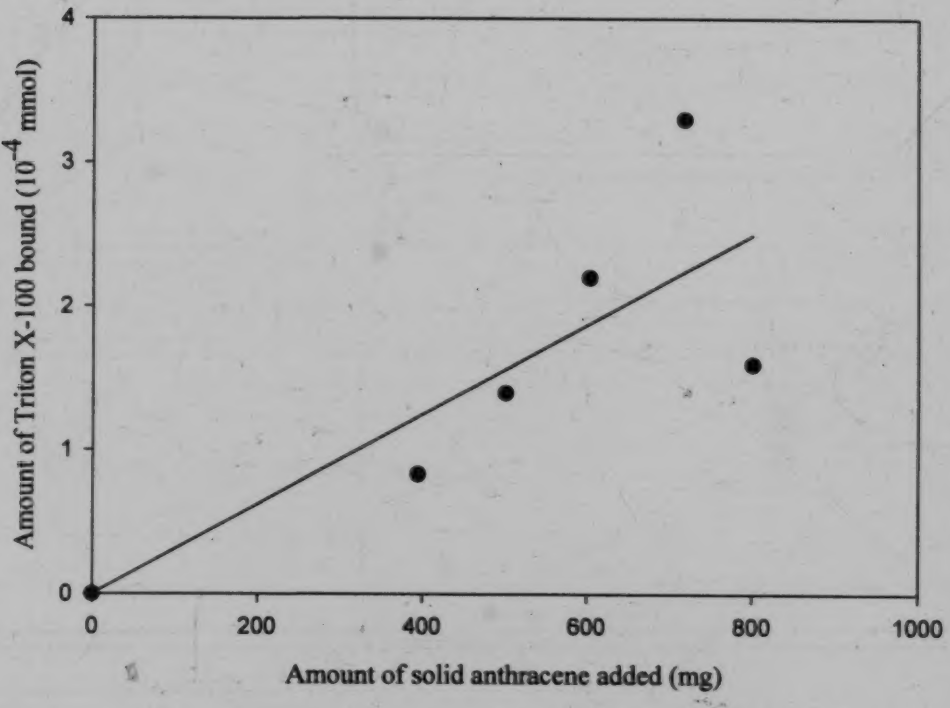
4.5.1 Sorption of Triton X-100 onto the surface of the anthracene particles

The data of Fig 4.14 showed that some Triton X-100 could be sorbed onto the surface of anthracene. A sample calculation is given in Appendix 4, which explains how the amount of sorbed Triton X-100 was determined from surface tension measurements. The data were scattered possibly due to the very low levels of sorption, which accounted for no more than 50% of the total Triton X-100 presented even when 700 mg anthracene were added, and possibly due to aggregation of anthracene particles. The slope of the straight line was determined to be 3.12×10^{-7} mmol/mg through linear regression. The amount of anthracene used in this study was only 50 mg, therefore, the amount of Triton X-100 sorbed onto the surface of anthracene would be $50\times 3.12\times10^{-7} = 1.56\times10^{-5}$ mmol.

For the cultures with the presence of Triton X-100 of 0.0048 mmol (0.18 CMC, 100 mL liquid culture) in this study, therefore, the amount bound to anthracene under

growth condition would be only $1.56\times10^{-5}/(4.8\times10^{-3})$ or 0.3% of the total surfactant. This order of magnitude estimate is not significantly affected by the scatter in the data of Fig 4.13. Even though only a small amount of Triton X-100 bound to the anthracene particles, the bound Triton X-100 covered a large portion of surface area of the anthracene particles, 67% in this study (The sample calculation was given in Appendix 4). The above is only an estimate of surface coverage, however, it still gives a sense of magnitude. The calculation of surface coverage was based on the following assumption: the hydrophobic tail of Triton X-100, a branched 8 carbon unit (Fig 3.1), was assumed to bind to the anthracene particles; sorption was assumed to be monolayer. It could also assume that Triton X-100 sorbed onto the anthracene particle with the end of its hydrophobic tail, which results in a different surface coverage.

Fig 4.14 Sorption of Triton X-100 onto the surfaces of the anthracene particles (Initial amount of Triton X-100 was $6.169*10^{-4}$ mmol)

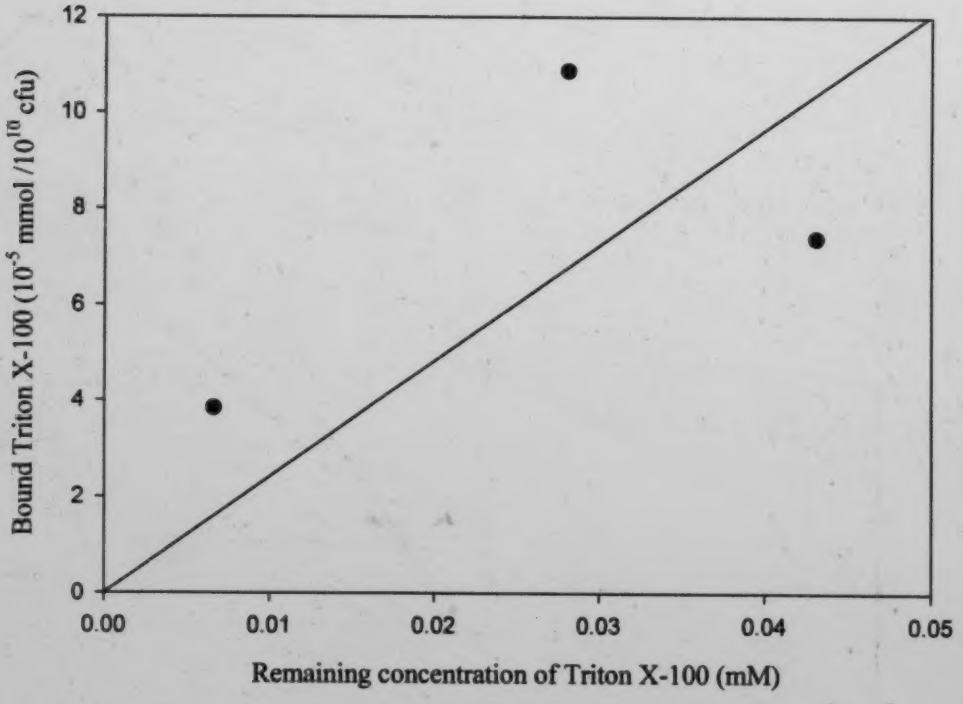


4.5.2 Sorption of Triton X-100 onto the *Mycobacterium* sp.

The data of Fig 4.15 showed that the amount of bound Triton increased with the concentration of Triton X-100 in the aqueous phase. The data were scattered possibly because the equilibrium partitioning of anthracene had not been reached. The data do allow an order of magnitude estimate of binding of surfactant. The slope of the straight line was determined to be 2.41×10^{-3} L/cfu. From this data, the amount of Triton X-100 bound to the *Mycobacterium* sp. could be estimated. For example, when the concentration of Triton X-100 was 0.18 CMC (0.048 mM), the maximum OD_{600} reading was around 0.6 in 100 ml of medium. Based on the above information, the bound Triton X-100 on the surfaces of the *Mycobacterium* sp. would be 7×10^{-4} mmol (Calculation is attached in Appendix 4). The ratio of Triton X-100 bound to the *Mycobacterium* sp. to Triton X-100 bound to the surfaces of anthracene was 70/1.56 = 45. Thus, although the scatter of the data of Fig 4.15 was large, the above calculation demonstrates that over 40 times as much Triton X-100 was bound to the *Mycobacterium* sp. as was bound to the anthracene. A rough calculation showed that in this study a large portion of surface area of the microorganism was covered by surfactant, approximately 36% (The sample calculation was given in Appendix 4). The calculation of surface coverage of cells followed the same assumption made for the calculation of surface coverage of the anthracene particles.

The above discussion leads to the following conclusions: (1) Triton X-100 is able to bind to both the *Mycobacterium* sp. and the surfaces of anthracene; (2) at least one order of magnitude more Triton X-100 was bound to the *Mycobacterium* sp. than the surfaces of the anthracene particles; (3) however, the amount of surface of the *Mycobacterium* sp. covered by surfactants was of the same order of magnitude as the coverage of the anthracene surface. Both surfaces had significant coverage.

Fig 4.15 Sorption of Triton X-100 on the surface of *Mycobacterium* sp.

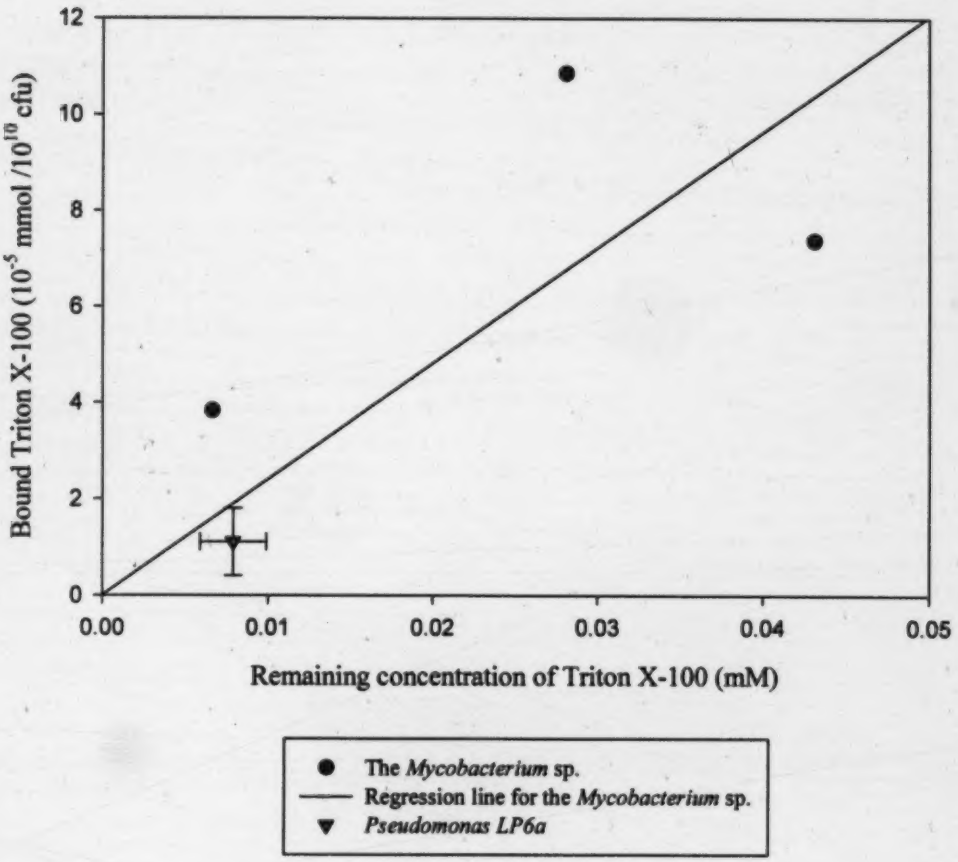


4.5.3 Sorption of Triton X-100 onto *Pseudomonas* LP6a

The data of Fig 4.16 showed that a large amount of Triton X-100 was bound to *Pseudomonas* LP6a, which accounted to more than 50 % of the total Triton X-100 added. *Pseudomonas* LP6a has the same kind of cell surface as the *Pseudomonas* sp. used for growing on anthracene in this study, therefore, they may have the same ability to bind Triton X-100. Consequently, the measurement of sorption of Triton X-100 onto *Pseudomonas* LP6a can give an estimate of Triton X-100 sorption onto the *Pseudomonas* sp. growing on anthracene. *Pseudomonas* LP6a grew poorly on anthracene, although it could grow on anthracene. Therefore, *Pseudomonas* LP6a previously grown on glucose was used in this series of experiments.

If 0.1 OD_{600} is assumed to correspond to a cell count of approximately 10^8 cfu/mL, the bound Triton X-100 per unit cell number could be estimated. The data was compared with the bound Triton X-100 per unit cell number by the *Mycobacterium* sp. (Fig 4.16). The data of Fig 4.16 showed that the sorption of Triton X-100 to either the *Mycobacterium* sp. or *Pseudomonas* LP6a was in the same level or the sorption to *Pseudomonas* LP6a was slightly lower. Further work may be of interest to identify the relationship between OD_{600} reading and cell number, then the cell surface coverage by Triton X-100 will be calculated.

Fig 4.16 Sorption of Triton X-100 on the surface of *Mycobacterium* sp. and *Pseudomonas* LP6a (Error bar shows the standard deviation from data for *Pseudomonas* LP6a)



4.6 Discussion of Inhibition of Microbial Growth on Anthracene

The experimental results of this study showed that the rate of the growth the *Mycobacterium* sp. and the *Pseudomonas* sp. on anthracene were inhibited by surfactants. Laha and Luthy (1991) proposed several mechanisms to explain the inhibition of microbial growth by surfactants: (1) surfactants are toxic to microorganisms; (2) micelles or micellized PAHs are toxic to microorganism; (3) surfactants are preferential substrates; (4) micellized substrates are not bioavailable or (5) surfactants may interfere with microbial adhesion. Consequently, we will assess each of these mechanisms in light of the experimental data.

4.6.1 Toxicity of Triton X-100 and Dowfax 8390

Triton X-100 was confirmed to be nontoxic to either the *Mycobacterium* sp. or the *Pseudomonas* sp., while Dowfax 8390 was toxic to the *Mycobacterium* sp. This observation is not surprising because nonionic surfactants are generally less toxic than ionic surfactants (Volkering et al., 1995). It was reported that ionic surfactant may cause the cell membrane to change irreversibly (Rouse et al., 1994). Wilson et al. (1995) compared the effects of Triton X-100 and two ionic surfactants, Dowfax 8390 and sodium dodecylbenzene sulfonate (SDBS), on biodegradation of benzene, toluene, ethylbenzene, xylenes and trimethylbenzenes dissolved in aqueous solution. They found that both ionic surfactants inhibited biodegradation, while Triton X-100 had no effects on biodegradation. They did not investigate the inhibition of degradation by ionic

surfactants, however, their results suggested that Triton X-100 was not toxic. Liu et al. (1994) showed that Triton X-100 was not toxic to *Escherichia coli* and mixed cultures, even at concentrations well above the CMC. They compared the growth of *Escherichia coli* on a soluble carbon source, glucose, with and without the presence of surfactants and observed no difference. This technique is exactly the same as used in this study. This simple technique allows a distinction between inhibition of uptake by surfactants from toxicity of surfactants. There are few studies of bioremediation involving Dowfax 8390, however, Stelmack et al. (1999) showed that neither Triton X-100 nor Dowfax 8390 were toxic to a *Pseudomonas* sp. The toxicity of Dowfax 8390 to the *Mycobacterium* sp. is anticipated to disappear if Dowfax 8390 is kept diluting to a concentration low enough. However, a very low concentration of surfactants is rarely used in soil bioremediation. Note that when a surfactant is toxic to one microorganism it does not necessarily mean it is toxic to other microorganisms, and vice versa.

4.6.2 Effect of Micelles

Some studies reported that surfactants below the CMC enhanced dissolution of some hydrocarbon compounds (Edwards et al, 1992; Grimberg et al., 1994), but the enhancement of solubility was very small and possibly due to experimental artifacts. Volkering et al. (1995) reported that Triton X-100 added below CMC had no effect on dissolution of naphthalene or phenanthrene. Since very low concentrations (0.1 CMC to 0.4 CMC) of surfactants were used in the present study, no micelles were present.

Without micelles, the possible toxicity of micelles or micellized PAHs is eliminated. Similarly, the bioavailability of PAHs in the micellar phase was not relevant.

4.6.3 Surfactants as Preferred Substrate

Control experiments which used Triton X-100 as the sole carbon source directly showed that Triton X-100 cannot be consumed by *Mycobacterium* sp. The data of Fig 4.7 and Fig 4.8 also indirectly confirmed that neither Triton X-100 nor Dowfax 8390 were consumed by the *Pseudomonas* species and that Triton X-100 was not consumed by the *Mycobacterium* species. If the bacteria consumed the surfactants, we would anticipate some difference between the growth curves of bacteria with or without the presence of surfactants. However, we did not observe this difference. Since both Triton X-100 and Dowfax 8390 are soluble and bacteria prefer soluble substrates, we should observe higher growth of the culture with either of the above surfactants than the control if the bacteria consumed the surfactant. However, this trend was not observed within the experimental period (14 days). Triton X-100 is generally not a preferred substrate in bioremediation. It was not biodegraded in many biodegradation processes (Breuil and Kushner, 1980; Liu et al., 1995; Volkering et al., 1995; Wilson et al., 1995). However, as discussed in §2.2.5, even if Triton X-100 were a preferential substrate, it would not necessarily mean that it will inhibit biodegradation (Providenti et al., 1993).

4.6.4 Interaction between Surfactants and Microorganisms

Anthracene was added as crystals. Due to its low solubility, most of the anthracene present in this study was in solid phase. Volkering et al. (1998) suggested that it is possible for microorganisms to facilitate the uptake of solid hydrocarbons via attachment to the solid hydrocarbon surfaces. Therefore in this study Triton X-100 may interfere with microbial adhesion, which resulted in inhibition of biodegradation by slowing the uptake of anthracene by the cells. Efroymson and Alexander (1991) observed that Triton X-100 interfered with the adsorption of *Arthrobacter* sp. to the HMN-water interface. Ortega-Calvo and Alexander (1994) reported the same observation. Stelmack et al. (1999) observed that both Triton X-100 and Dowfax 8390 interfered with the adhesion of either a *Mycobacterium* sp. or a *Pseudomonas* sp. to NAPLs from contaminated soils. In the same study, they also reported that both surfactants were able to remove the previously bound *Mycobacterium* sp. or *Pseudomonas* sp. from the surfaces. The present study reinforces Stelmack's observation that adhesion may be significant for degrading of solid hydrocarbons.

Neu (1996) suggested that surfactants could change microbial adhesion by adsorbing to the surfaces of microorganism, or to the surfaces of hydrocarbon, or both. This study showed that Triton X-100 could bind to the *Mycobacterium* sp., *Pseudomonas* LP6a and the anthracene particles. Even though the amount of Triton X-100 bound to the *Mycobacterium* sp. was much more than that to anthracene (at least one order of magnitude higher), the percentage of surface coverage of cells by Triton X-100 was in the same magnitude as that of anthracene. A considerable portion of either cell surface and anthracene surface were covered by Triton X-100. Therefore, it is likely that the sorption of Triton X-100 on the surfaces changes the surface hydrophobicity, making the

cells and the anthracene particles more hydrophilic. This change in surface property would make it more difficult for cells to adhere to the surface of anthracene. The reduction in the interfacial tension caused by the living cells in this study was only equivalent to addition of 0.012 mmol Triton X-100 in 1 L liquid culture (0.05 CMC). This observation suggested that the effect of any biosurfactant production was very small.

In this study, the cells had a much larger surface area than the surface area of the anthracene particles. The initial inoculum in this study was usually around 0.07 OD_{600}, which was the lowest cell density within the growth period. At this density, the effective cell surface for adhering to the anthracene particle was 7×10^{-3} m^2, however, the surface area that the anthracene particle could provide for adhesion was only 2.5×10^{-3} m^2. Therefore the cells were much too numerous to all adhere to the anthracene particle. Observation of the cells showed that they were well dispersed in the liquid phase during growth. These two points suggest that adhesion was transient - cells attach and detach continuously. This hypothesis is consistent with the work of Tongpim and Pickard (1996) who found that an adhered biofilm was only established when mixing of the culture was minimized. If transient adhesion played a major role in taking up anthracene by cells, then surfactants would inhibit cell growth on solid anthracene and consequently inhibit biodegradation of anthracene (Stelmack et al., 1999).

Following this idea of transient adhesion, it is of interest for further work to determine the dissolution rate of anthracene in medium without the presence of microorganism and compare this rate with uptake rate by the microorganism. If the dissolution rates were much lower than the uptake rate, then it would indicate microbial adhesion, which provides a close contact between cells and anthracene, facilitating

microbial growth on anthracene. The possible reason is that cell wall components may act as micelles to facilitate dissolution and uptake of anthracene.

The above hypothesis could also explain the partial saturation of inhibition. The sorption of surfactant to both the microorganism and the anthracene particles would reduce the surface hydrophobicity, which would in turn interfere with the transient adhesion and consequently inhibit microbial growth on anthracene. Therefore after the hydrophobic surfaces had been completely covered by surfactant, the hydrophobicity of both surfaces would not further change with addition of more surfactant, which would result in the inhibition reached saturation. As discussed in §4.2.2, the surface of the *Pseudomonas* sp. is less hydrophobic than that of the *Mycobacterium* sp, therefore, it would be more easily saturated at low surfactant concentration and hence more sensitive to addition of surfactants.

In the sorption experiments, the absorption of Triton X-100 was measured after shaking the mixture of Triton X-100 and liquid culture for only 1 hour, however, a high sorption of surfactant was still obtained. This indirectly confirmed that in this study the absorption occurred quickly. Huysman and Verstraete (1993) reported that microbial adhesion could occur within 20 minutes, therefore, all of the surface interactions would likely occur quickly and respond rapidly to changes in concentration.

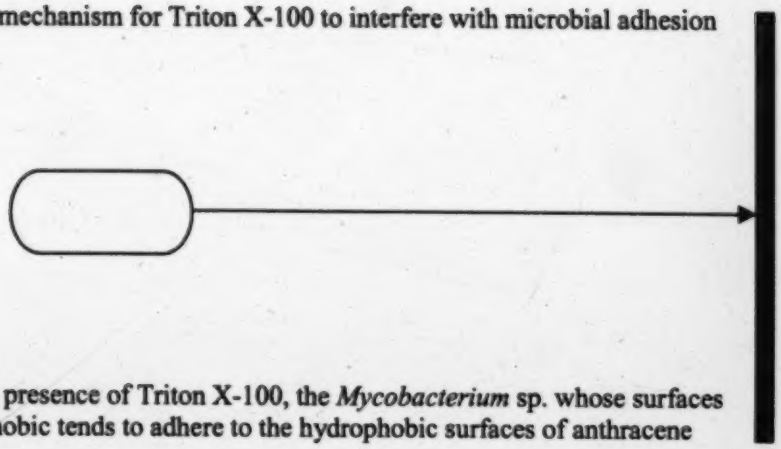
Since sorption of surfactants to either microorganisms or hydrocarbons is a physical interaction, it should be completely reversible. In this study we observed that the biodegradation rate could be further decreased by addition of more Triton X-100, and it also could be completely recovered by dilution with fresh medium. The rate of recovery was rapid at concentrations of surfactant below CMC. Laha and Luthy (1991 and 1992)

also reported that dilution of surfactant resulted in recovery of biodegradation of phenanthrene by mixed cultures. However, they did not add more surfactants to examine the possible further inhibition and nor did they show that the reversibility was complete or partial. This study was able to show that effects of surfactant on microbial growth on anthracene were completely reversible, no matter by addition or dilution of surfactant. Laha and Luthy (1991 and 1992) observed no lag phase after dilution. However, in this study, the recovery of microbial growth by dilution of surfactants indicated a possible lag phase (Figure 4.10 and Figure 4.11), therefore, it might be of interest for further work to examine the dependence of recovery of microbial growth on the surfactant concentrations before and after dilution. Laha and Luthy (1991 and 1992) investigated the effects of surfactants on biodegradation of phananthrene by mixed culture. This study complements theirs by examining the effects of surfactants on the microbial growth on anthracene under well controlled condition.

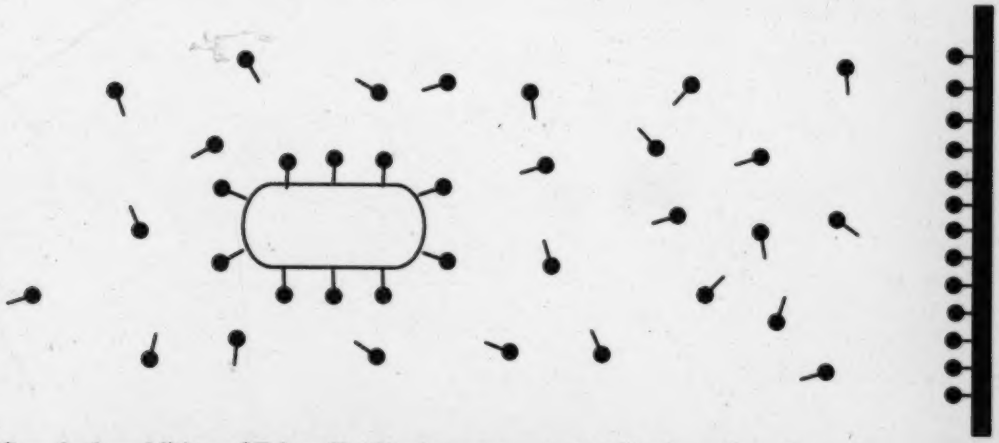
The five proposed mechanisms to explain the inhibition of surfactants on microbial growth have been assessed. The experimental results from this study were able to rule out mechanisms (1) through (4). Therefore the possible mechanism would be that surfactants interfere with microbial adhesion to anthracene and subsequently interfere with growth (Fig 4.15).

Surfactants have been proposed to enhance bioremediation due to their solubilizing ability. However, this study showed that even at very low concentrations, the nonionic surfactant Triton X-100 inhibited biodegradation of anthracene and probably

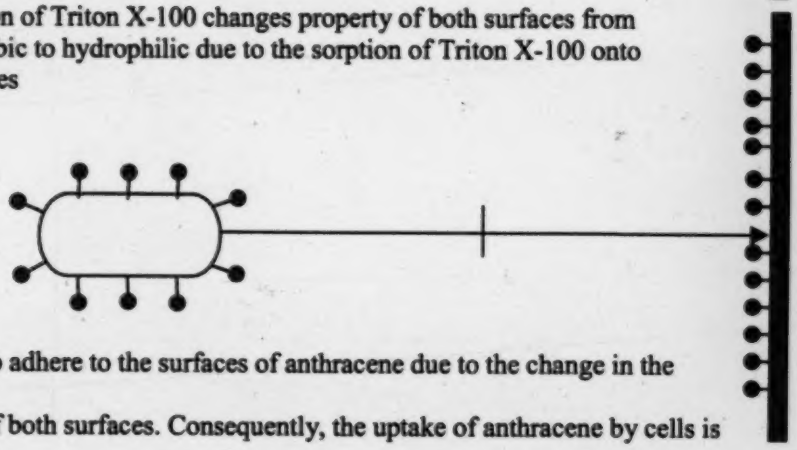
Fig 4.17 Proposed mechanism for Triton X-100 to interfere with microbial adhesion



Step 1: without the presence of Triton X-100, the *Mycobacterium* sp. whose surfaces are hydrophobic tends to adhere to the hydrophobic surfaces of anthracene



Step 2: the addition of Triton X-100 changes property of both surfaces from hydrophobic to hydrophilic due to the sorption of Triton X-100 onto the surfaces



Step 3: cell fails to adhere to the surfaces of anthracene due to the change in the property of both surfaces. Consequently, the uptake of anthracene by cells is inhibited

inhibited the adhesion of microorganisms to the target compound. Consequently the application of surfactants to bioremediation should be based on a comprehensive consideration of its solubilizing ability and possible inhibition of microbial adhesion. The conflicting results of effects of surfactants on bioremediation reported in the literature are probably due to the balance between an enhancement of dissolution of hydrocarbons and inhibition of microbial adhesion.

CHAPTER 5 CONCLUSIONS AND RECOMMENDATIONS

The critical micelle concentrations of Triton X-100 and Dowfax 8390 in the growth medium were determined to be 0.26 mM and 0.48 mM, respectively. These data determined the selection of surfactant concentrations.

Triton X-100 inhibited the rate and possibly the extent of either the *Mycobacterium* sp. or the *Pseudomonas* sp. on anthracene, even at very low concentrations (0.024 mM or 0.09 CMC). However, inhibition of microbial growth exhibited partial saturation when the concentration of Triton X-100 was over 0.048 mM (0.18 CMC). Below this concentration, the growth rate of the *Mycobacterium* sp. on anthracene decreased rapidly with the increase of concentration of Triton X-100. Above this concentration, the addition of more Triton X-100 had less effect on microbial growth. Fewer data were collected for the *Pseudomonas* sp., therefore, the evidence for possible partial saturation was not clear. However, the *Pseudomonas* sp. was much sensitive to Triton X-100 than the *Mycobacterium* sp., which was possibly due to the difference in cell surface hydrophobicity.

Further studies may use various types of surfactants and microorganisms to examine whether this observation of partial saturation is a general trend and the correlation between cell surface hydrophobicity and inhibition of microbial growth by surfactants.

The growth of the *Mycobacterium* sp. on anthracene showed linear growth with and without the presence of surfactants while the growth of the *Pseudomonas* sp. on

anthracene showed biphasic growth. In both cases microbial growth on anthracene was limited by its dissolution rate, as indicated by linear growth.

Although previous work showed that biodegradation was recovered by dilution of surfactant, the results of this study was able to show that the inhibition of microbial growth could be increased by addition of more Triton X-100 and also could be recovered by dilution of surfactants present. Both addition and dilution of surfactant were completely reversible. Further inhibition occurred immediately after the addition of more Triton X-100. The rate of recovery was also quite rapid at surfactant concentrations below the CMC. This observation indicated that the interaction between Triton X-100 and microorganism / anthracene was very fast.

Further studies may show how fast the rate of recovery is at surfactant concentrations above the CMC, with the use of an initial inoculum concentration high enough to avoid any lag phase.

When glucose was used as the carbon source, the growth of either the *Mycobacterium* sp. or the *Pseudomonas* sp. showed no inhibition in the presence of Triton X-100 at a concentration below the CMC. This observation indicated that Triton X-100 was not toxic to either the *Mycobacterium* sp. or the *Pseudomonas* sp. However, by the same technique, Dowfax 8390 was determined to be toxic to *Mycobacterium* sp. It indicates that the toxicity of surfactant is strain-dependent. Triton X-100 was also determined not to be a preferential substrate to anthracene. The concentrations of both surfactants used in this study were below CMC, therefore, the possible effect of micelles was obviated.

This study showed that Triton X-100 was able to bind to both the *Mycobacterium* sp. and the anthracene particles. Even though the amount of surfactant bound to the *Mycobacterium* sp. was much more significant (at least an order of magnitude higher) than that to anthracene, the coverage of both the surface of the *Mycobacterium* sp. and the surface of anthracene particles by Triton X-100 was high and in the same order of magnitude. This study also showed that Triton X-100 was able to bind to the *Pseudomonas* LP6a. Due to the similarity of the cell surface properties of *Pseudomonas* sp., the above observation indicated that Triton X-100 would be able to bind to the *Pseudomonas* sp. used to grow on anthracene in this study.

Further studies may show the possible relationship between the coverage by surfactant and the saturation of inhibition of microbial growth.

The data from this study were consistent with the hypothesis that surfactants interfered with the adhesion of the microorganisms to the surfaces of hydrophobic compounds, and consequently inhibited the microbial growth on these hydrophobic compounds. The possible mechanism is hypothesized as follows: surfactants binds to the surfaces of both microorganism and hydrophobic compounds. Surfactants altered the hydrophobicity of the surfaces by sorption of their hydrophobic tail to the hydrophobic surfaces and keeping their hydrophilic head pointing outwards, which results in a change of surface hydrophobicity from hydrophobic to hydrophilic. This alteration of surface property prevents the microorganism from adhering to the previously hydrophobic compounds. If transient adhesion is a important factor for uptaking hydrophobic compounds, then the interference with this adhesion would cause inhibition of microbial growth on the hydrophobic compounds.

Further work needs to be conducted to determine the role of transient adhesion in uptake of hydrocarbon compounds that are present in highly viscous or solid phases.

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Appendix 1 Raw Data

Table A.1 CMC of Triton X-100 in growth medium at 21 °C

Surfactant Concentration (mM)	Apparent Surface Tension (dynes/cm) - Run 1	Apparent Surface Tension (dynes/cm) - Run 2	Average Apparent Surface Tension (dynes/cm)	Absolute Surface Tension (dynes/cm)
0.116	38.3	38.7	38.5	34.5
0.162	37.3	37.0	37.2	33.3
0.186	36.2	36.6	36.4	32.6
0.209	35.6	35.4	35.5	31.7
0.232	35.0	34.4	34.7	31.0
0.255	34.2	34.0	34.1	30.4
0.278	34.0	33.9	34.0	30.3

Table A.2 CMC of Dowfax 8390 in growth medium at 21 °C

Surfactant Concentration (mM)	Apparent Surface Tension (dynes/cm) - Run 1	Apparent Surface Tension (dynes/cm) - Run 2	Average Apparent Surface Tension (dynes/cm)	Absolute Surface Tension (dynes/cm)
0.1	53.0	52.7	52.9	48.3
0.2	51.4	51.5	51.5	46.9
0.4	50.6	50.9	50.8	46.3
0.6	50.5	50.4	50.5	46.0
0.8	50.6	50.2	50.4	45.9
1.0	50.4	50.4	50.4	45.9
1.2	50.3	50.4	50.4	45.9

The absolute surface tension was calculated by

$$S = P \times F$$

where:

F: the correction factor

R: the radius of the ring, cm

r: the radius of the wire of the ring, cm

P: the apparent value or dial reading, dynes/cm

D: the density of the lower phase, g/cm^3

d: the density of the upper phase, g/cm^3

R/r: 53.0322

C: the circumference of the ring, 5.920 cm

Table A.3 Growth of *Mycobacterium* sp. on anthracene with Triton X-100 at different concentrations - series 1

	Day	0	1	2	3	5	7	10	14
Control	A	0.065	0.109	0.220	0.303	0.453	0.562	0.705	0.711
	B	0.064	0.110	0.221	0.302	0.442	0.571	0.717	0.848
	C	0.072	0.109	0.213	0.294	0.435	0.565	0.729	0.850
	D	0.067	0.105	0.204	0.292	0.429	0.563	0.725	0.828
	Avg.	0.067	0.108	0.215	0.298	0.440	0.565	0.719	0.809
	σ	0.004	0.002	0.008	0.006	0.010	0.004	0.011	0.066
0.09 CMC	A	0.075	0.161	0.242	0.312	0.435	0.534	0.660	0.726
	B	0.080	0.156	0.233	0.310	0.440	0.550	0.646	0.708
	C	0.075	0.157	0.231	0.297	0.399	0.515	0.685	0.748
	D	0.078	0.158	0.236	0.311	0.440	0.548	0.661	0.715
	Avg.	0.077	0.158	0.236	0.308	0.429	0.537	0.663	0.724
0.18 CMC	A	0.081	0.144	0.212	0.279	0.373	0.482	0.611	0.674
	B	0.082	0.157	0.226	0.285	0.382	0.460	0.577	0.656
	Avg.	0.082	0.151	0.219	0.282	0.378	0.471	0.594	0.665
0.27 CMC	A	0.094	0.160	0.215	0.274	0.361	0.413	0.521	0.612
	B	0.090	0.152	0.204	0.265	0.338	0.409	0.515	0.574
	C	0.086	0.143	0.203	0.262	0.349	0.428	0.565	0.633
	Avg.	0.090	0.152	0.207	0.267	0.349	0.417	0.534	0.606
0.36 CMC	A	0.103	0.154	0.218	0.269	0.344	0.406	0.517	0.563
	B	0.104	0.158	0.212	0.262	0.336	0.392	0.498	0.577
	Avg.	0.104	0.156	0.215	0.266	0.340	0.399	0.508	0.570

Table A.4 Growth of *Mycobacterium* sp. on anthracene with Triton X-100 at different concentrations - series 2

	Day	0	1	3	4	6	7	10	14
Control	A	0.060	0.152	0.320	0.401	0.532	0.594	0.718	0.576
	B	0.070	0.138	0.311	0.402	0.534	0.600	0.697	0.620
	C	0.064	0.134	0.305	0.394	0.508	0.574	0.632	0.589
	Avg.	0.065	0.141	0.312	0.399	0.525	0.589	0.682	0.595
	σ	0.005	0.009	0.008	0.004	0.014	0.014	0.045	0.023
0.09 CMC	A	0.078	0.104	0.205	0.275	0.392	0.463	0.649	0.641
	B	0.094	0.123	0.222	0.310	0.426	0.481	0.570	0.610
	Avg.	0.086	0.114	0.214	0.293	0.409	0.472	0.610	0.626
0.18 CMC	A	0.096	0.098	0.153	0.191	0.268	0.326	0.435	0.504
	B	0.097	0.111	0.158	0.208	0.272	0.321	0.435	0.530
	Avg.	0.097	0.105	0.156	0.200	0.270	0.324	0.435	0.517
0.27 CMC	A	0.095	0.112	0.159	0.190	0.270	0.321	0.442	0.508
	B	0.095	0.106	0.169	0.220	0.291	0.344	0.483	0.524
	Avg.	0.095	0.109	0.164	0.205	0.281	0.333	0.463	0.516

Table A.5 Growth of *Mycobacterium* sp. on anthracene with Triton X-100 at different concentrations - series 3

	Day	0	1	2	3	5	7	10	14
Control	A	0.046	0.161	0.241	0.346				
	B	0.049	0.193	0.272	0.366				
	C	0.050	0.160	0.237	0.341				
	D	0.041	0.190	0.286	0.401				
	Avg.	0.047	0.176	0.259	0.364				
0.09 CMC	A	0.045	0.148	0.206	0.247	0.370	0.483	0.645	0.653
	B	0.045	0.152	0.202	0.247	0.377	0.498	0.610	0.620
	Avg.	0.045	0.150	0.204	0.247	0.374	0.491	0.628	0.637
0.14 CMC	A	0.047	0.142	0.184	0.237	0.332	0.425		
	B	0.049	0.151	0.187	0.238	329.000	0.419		
	Avg.	0.048	0.147	0.186	0.238	0.331	0.422		
0.16 CMC	A	0.047	0.132	0.165	0.203	0.280	0.348	0.455	0.461
	B	0.049	0.133	0.165	0.204	0.293	0.373	0.455	0.465
	Avg.	0.048	0.133	0.165	0.204	0.287	0.361	0.455	0.463
0.18CMC	A	0.051	0.131	0.155	0.192	0.271	0.337	0.437	0.447
	B	0.050	0.135	0.162	0.201	0.282	0.362	0.464	0.478
	C	0.054	0.138	0.169	0.210	0.299	0.370	0.495	0.500
	D	0.051	0.134	0.174	0.212	0.298	0.375	0.478	0.485
	Avg.	0.052	0.135	0.165	0.204	0.288	0.361	0.469	0.478
	σ	0.002	0.004	0.008	0.009	0.013	0.017	0.025	0.022
0.27 CMC	A	0.054	0.150	0.195	0.242	0.322	0.406	0.527	0.532
	B	0.057	0.155	0.193	0.243	0.338	0.409	0.520	0.536
	Avg.	0.056	0.153	0.194	0.243	0.330	0.408	0.524	0.534
0.36 CMC	A	0.058	0.140	0.160	0.180	0.275	0.341	0.442	0.453
	B	0.056	0.134	0.154	0.189	0.281	0.338	0.440	0.447
	Avg.	0.057	0.137	0.157	0.185	0.278	0.340	0.441	0.450

Table A.6 Growth rate (ΔOD_{600}/Day) of *Mycobacterium* sp. on anthracene with Triton X-100 at different concentrations

	Concentration of Triton X-100 (CMC)	Growth Rate	Error (ΔOD_{600}/Day)
Series 1	0	0.070	0.003
	0.09	0.060	0.002
	0.18	0.050	0.002
	0.27	0.041	0.003
	0.36	0.036	0.003
Series 2	0	0.068	
	0.09	0.063	
	0.18	0.041	
	0.27	0.041	
Series 3	0.09	0.058	
	0.14	0.047	
	0.16	0.039	
	0.18	0.039	
	0.27	0.042	
	0.36	0.038	
Reversibility Study	0	0.063	
	0.05	0.068	
	0.27	0.037	
	1.35	0.016	0.001
	0.27	0.034	
	0.03	0.065	

Table A.7 Growth of *Mycobacterium* sp. on anthracene with Dowfax 8390 at different concentrations

	Day	0	1	2	3	4	7
Control	A	0.068	0.098	0.166	0.240	0.307	0.574
	B	0.068	0.101	0.170	0.240	0.311	0.565
	Avg.	0.068	0.100	0.168	0.240	0.309	0.570
0.17 CMC	A	0.071	0.084	0.085	0.080	0.082	0.084
	B	0.066	0.076	0.076	0.080	0.077	0.074
	Avg.	0.069	0.080	0.081	0.080	0.080	0.079
0.83 CMC	A	0.068	0.085	0.091	0.080	0.084	
	B	0.068	0.091	0.104	0.107	0.109	
	Avg.	0.068	0.088	0.098	0.094	0.097	

Table A.8 Growth of *Pseudomonas* sp. on anthracene with Triton X-100 at different concentrations

	Day	0	0.5	1	2	4	5	8
Control	A	0.064	0.159	0.206	0.213	0.276	0.295	0.360
	B	0.063	0.146	0.190	0.196	0.257	0.304	0.344
	Avg.	0.064	0.153	0.198	0.205	0.267	0.300	0.352
0.023 CMC	A	0.066	0.116	0.158	0.183	0.243	0.269	0.317
	B	0.065	0.117	0.165	0.195	0.260	0.276	0.326
	Avg.	0.066	0.117	0.162	0.189	0.252	0.273	0.322
0.046 CMC	A	0.065	0.100	0.143	0.159	0.197	0.215	0.243
	B	0.067	0.123	0.179	0.210	0.254	0.286	0.298
	Avg.	0.066	0.112	0.161	0.185	0.226	0.251	0.271
0.069 CMC	A	0.071	0.092	0.122	0.138	0.161	0.171	0.178
	B	0.070	0.090	0.118	0.125	0.166	0.180	0.191
	Avg.	0.071	0.091	0.120	0.132	0.164	0.176	0.185
0.09 CMC	A	0.070	0.072	0.099	0.107	0.134	0.156	0.183
	B	0.071	0.077	0.093	0.105	0.125	0.144	0.163
	Avg.	0.071	0.075	0.096	0.106	0.130	0.150	0.173

Table A.9 Growth rate (ΔOD_{600}/Day) of *Pseudomonas* sp. on anthracene with Triton X-100 at different concentrations

Concentration of Triton X-100 (CMC)	Growth Rate (ΔOD_{600}/Day)	Error (ΔOD_{600}/Day)
0	0.032	0.0004
0.023	0.029	0.0013
0.046	0.022	0.0006
0.069	0.014	0.0006
0.09	0.013	0.0012

Table A.10 Growth of *Mycobacterium* sp. on glucose with either Triton X-100 or Dowfax 8390 below CMC

	Day	0	1	2	3	4	5	6	7
Control	A	0.094	0.158	0.235	0.339	0.486	0.707	0.839	0.619
	B	0.099	0.152	0.245	0.325	0.494	0.713	0.840	0.578
	C	0.097	0.149	0.218	0.322	0.469	0.661	0.826	0.690
	D	0.098	0.153	0.241	0.342	0.510	0.734	0.849	0.690
	Avg.	0.097	0.153	0.235	0.332	0.490	0.704	0.839	0.644
Triton X-100	A	0.096	0.184	0.242	0.337	0.464	0.680	0.824	0.774
	B	0.098	0.171	0.255	0.356	0.512	0.726	0.769	0.731
	C	0.096	0.178	0.250	0.353	0.517	0.736	0.778	0.730
	D	0.086	0.173	0.254	0.356	0.505	0.697	0.760	0.734
	Avg.	0.094	0.177	0.250	0.351	0.500	0.710	0.783	0.742
Dowfax 8390	A	0.090	0.094	0.087	0.085				
	B	0.095	0.105	0.100	0.095				
	C	0.090	0.130	0.128	0.124				
	D	0.083	0.108	0.100	0.094				
	Avg.	0.090	0.109	0.104	0.100				

Table A.11 Growth of *Pseudomonas* sp. on glucose with Triton X-100 at 0.45 CMC

	Hour	0	2	4	6	8	10	12	18
Control	A	0.073	0.116	0.208	0.416	0.737	0.724	0.710	0.630
	B	0.072	0.120	0.218	0.415	0.753	0.733	0.721	0.643
	C	0.073	0.115	0.212	0.409	0.739	0.719	0.711	0.630
	D	0.075	0.127	0.224	0.422	0.778	0.747	0.723	0.647
	Avg.	0.073	0.120	0.216	0.416	0.752	0.731	0.716	0.638
Triton X-100	A	0.077	0.135	0.225	0.411	0.690	0.736	0.715	0.629
	B	0.080	0.137	0.229	0.404	0.697	0.735	0.718	0.627
	C	0.079	0.138	0.233	0.415	0.686	0.734	0.727	0.639
	D	0.081	0.136	0.224	0.409	0.682	0.720	0.706	0.622
	Avg.	0.079	0.137	0.228	0.410	0.689	0.731	0.717	0.629

Table A.12 Growth of *Mycobacterium* sp. on anthracene with changing concentration of Triton X-100

(1) Control

Day	0	1	2	3	5	7
A	0.062	0.115	0.195	0.276	0.387	0.485
B	0.057	0.121	0.212	0.291	0.424	0.522
C	0.058	0.115	0.194	0.262	0.391	0.484
Avg.	0.059	0.117	0.200	0.276	0.401	0.497

(2) Addition of more surfactants (0.05 CMC to 0.27 CMC after 4 days)

Day	0	1	2	3	4	5	6	7
A	0.080	0.134	0.196	0.264	0.321	0.356	0.387	0.414
B	0.086	0.110	0.194	0.262	0.334	0.374	0.419	0.460
Avg.	0.083	0.122	0.195	0.263	0.328	0.365	0.403	0.437

(3) Dilution 1 – Dilution of surfactants from 1.35 CMC to 0.14 CMC

	Day	0	1	2	3	4	5	6	7
1.5 CMC	A	0.086	0.091	0.104	0.116	0.142	0.160	0.156	0.171
	B	0.088	0.092	0.106	0.117	0.135	0.152	0.132	0.130
	Avg.	0.087	0.092	0.105	0.117	0.139	0.156	0.144	0.151
0.15 CMC	A					0.033	0.052	0.060	0.090
	B					0.032	0.050	0.060	0.083
	Avg.					0.033	0.051	0.060	0.087

(4) Dilution 2 – Dilution of surfactants from 0.27 CMC to 0.03 CMC

	Day	0	1	2	3	4	5	6	7	8	9
0.3 CMC	A	0.119	0.126	0.160	0.201	0.239	0.266	0.304	0.341	0.371	
	B	0.118	0.116	0.144	0.179	0.212	0.238	0.268	0.295	0.325	
	C	0.117	0.115	0.147	0.196	0.236	0.272	0.306	0.342	0.374	
	Avg.	0.118	0.119	0.150	0.192	0.229	0.259	0.293	0.326	0.357	
0.03 CMC	A						0.031	0.062	0.111	0.164	0.217
	B						0.029	0.080	0.149	0.215	0.290
	C						0.033	0.075	0.134	0.214	0.287
	Avg.						0.031	0.072	0.131	0.198	0.265

Table A.13 Surface tension of the growth medium as a method for the determination of sorption of Triton X-100 onto surfaces

(1) Sorption to the surface of the anthracene particles

Amount of Anthracene Added (mg)	Apparent Surface Tension (dynes/cm) - Run 1	Apparent Surface Tension (dynes/cm) - Run 2	Average Surface Tension (dynes/cm)
0	48.6	48.8	48.7
394.5	49.6	49.7	49.65
501.2	50.5	50.2	50.35
603.2	51.5	51.7	51.6
718.0	53.6	54	53.8
800.5	51	50.3	50.65

The volume of the growth medium for this series of experiments was 25 mL.

(2) Sorption to the *Mycobacterium* sp.

Initial apparent surface tension (dynes/cm) - Run 1	50.5	43.0	43.0
Initial apparent surface tension (dynes/cm) - Run 2	50.0	43.2	43.2
Average of the initial apparent surface tension (dynes/cm)	50.3	43.1	43.1
Initial concentration of free Triton X-100, C_0 (mM)	0.0112	0.0581	0.0581
Final apparent surface tension (dynes/cm) - Run 1	57.5	45.2	47.6
Final apparent surface tension (dynes/cm) - Run 2	57.1	44.9	48.1
Average of the final apparent surface tension (dynes/cm)	57.3	45.1	47.9
Final concentration of free Triton X-100, C_1 (mM)	0.0086	0.0431	0.0281
Volume of medium, V (mL)	20	25	25
Amount of Bound Triton X-100, $N=V*(C_0-C_1)$ (mmol)	0.000051	0.000374	0.000749
OD_{600} reading	0.894	0.634	0.69
Volume of liquid culture, V_L (mL)	75	80	100
Amount of Cells, $Nc=V_L*OD_{600}/0.1*10^8$ (cfu)	6.71E+10	5.07E+10	6.90E+10
Bound Triton X-100, N/Nc (10^{-5}mmol/10^{10} cfu)	0.76	7.38	10.86

(3) Sorption to *Pseudomonas* LP6a

Initial apparent surface tension (dynes/cm) - Run 1	53.7	53.7	53.7
Initial apparent surface tension (dynes/cm) - Run 2	54.0	54.0	54.0
Average of the initial apparent surface tension (dynes/cm)	53.9	53.9	53.9
Initial concentration of free Triton X-100, C_0 (mM)	0.0112	0.0112	0.0112
Final apparent surface tension (dynes/cm) - Run 1	55.5	58.2	54.7
Final apparent surface tension (dynes/cm) - Run 2	55.7	58.3	55.2
Average of the final apparent surface tension (dynes/cm)	55.6	58.3	55.0
Final concentration of free Triton X-100, C_1 (mM)	0.0086	0.0057	0.0095
Volume of medium, V (mL)	25	25	25
Amount of Bound Triton X-100, $N=V*(C_0-C_1)$ (mmol)	0.000066	0.000138	0.000043
OD_{600} reading	0.758	0.774	0.771
Volume of liquid culture, V_L (mL)	100	94	100
Amount of Cells, $Nc=V_L*OD_{600}/0.1*10^8$ (cfu)	7.58E+10	7.28E+10	7.71E+10
Bound Triton X-100, N/Nc (10^{-5}mmol/10^{10} cfu)	0.87	1.9	0.56

Appendix 2 Standard Curve to Relate the Surface Tension of the Growth Medium to Concentrations of Triton X-100

This standard curve was used to calculate the adsorption of Triton X-100 onto the surfaces of both microorganisms and the anthracene particles. To obtain the standard curve, a number of solutions, each of which contained Triton X-100 at different concentrations ranging from 0 to 0.18 CMC (0 to 0.048 mM) in 25 ml growth medium were prepared. Then the surface tension of each solution was measured. The measurements of these surface tensions were plotted against the concentrations of Triton X-100 (Fig A.1).

From Fig A.1, an equation (eq. 7) could be obtained as follow:

Surface tension (dynes/cm) = 33.81 – 15.07 × log(Concentration of Triton X-100 (CMC))

This equation was used in the calculations in absorption experiments.

Appendix 3 The Repeat Experiments of the Effects of Triton X-100 on the Growth of the *Mycobacterium* sp. on Anthracene

Fig A.2 and Fig A.3 show the results of the repeat experiments on the effects of Triton X-100 on the biodegradation of anthracene by the *Mycobacterium* sp. The slopes of the linear growth section of each experiment were calculated through linear regression and are reported in Fig 4.4.

Fig A.2 Growth of *Mycobacterium* sp. on anthracene with the addition of Triton X-100 - Series 2 (0.09 CMC to 0.27 CMC (0.024 mM to 0.072 mM), error bar show the standard deviation based on three replicate)

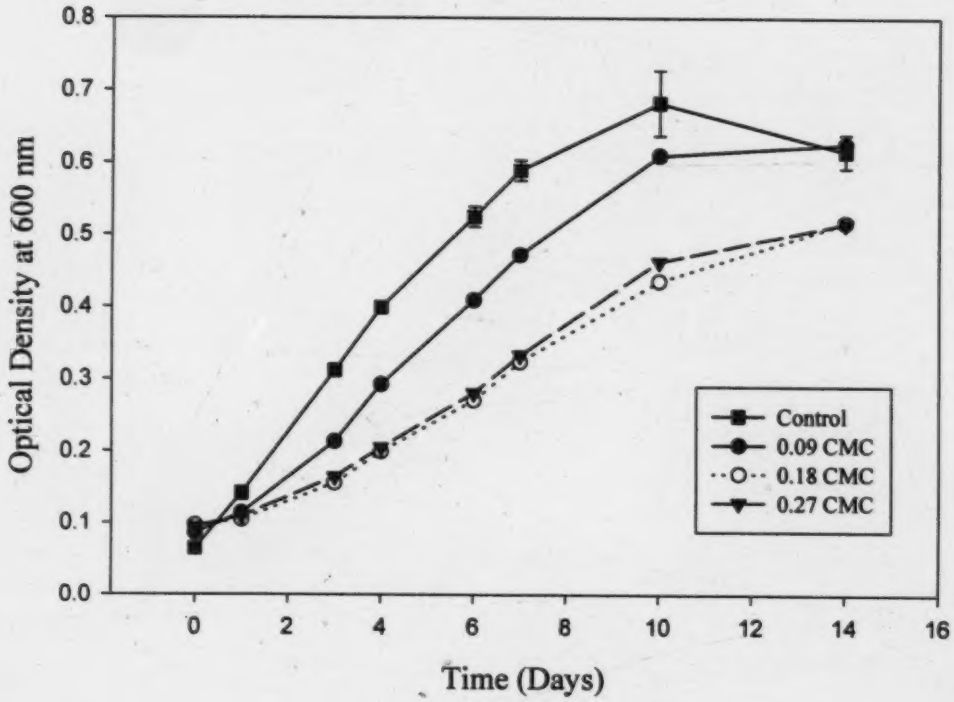
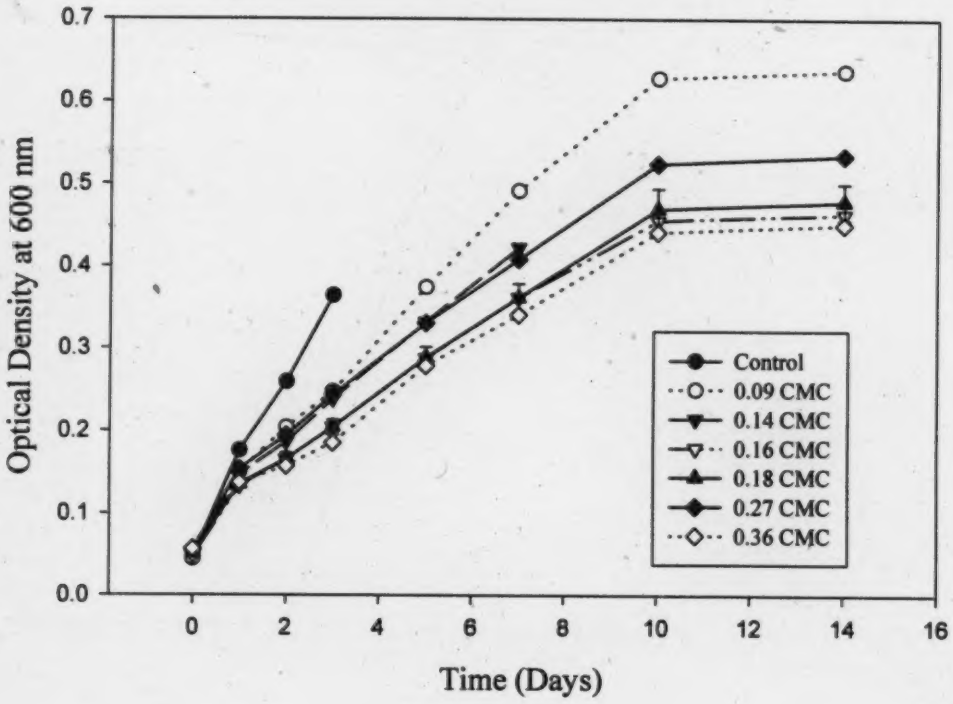


Fig A.3 Growth of *Mycobacterium* sp. on anthracene with the addition of Triton X-100 - Series 3 (0.09 CMC to 0.36 CMC (0.024 mM to 0.096 mM), error bars show the standard deviation based on four replicates)



Appendix 4 Sample Calculations

(1) Obtaining the amount of Triton X-100 bound to anthracene through the measurements of surface tension

When 394.5 mg anthracene was added into the 25 ml growth medium which contained a certain amount of Triton X-100, the apparent surface tension of that medium increased from 48.70 dynes/cm (T_0) to 49.65 dynes/cm (T_1) . This increase in surface tension meant some Triton X-100 was bound by anthracene. Following is a sample calculation to get the amount of bound Triton X-100 based on the above data.

According to eq. 7, the initial concentration of Triton X-100 in the medium was:

$C_0 = 10^{(33.81-48.70)/15.07} = 0.103$ CMC;

And the final concentration of free Triton X-100 in the medium was:

$C_1 = 10^{(33.81-49.65)/15.07} = 0.089$ CMC;

Therefore the amount of bound Triton X-100 was:

$N = (C_0 - C_1) \times 0.24$ mmol/l $\times 0.025 = 8.3 \times 10^{-5}$ mmol

The amount of Triton X-100 bound by the *Mycobacterium* sp. and the *Pseudomonas* LP6a was obtained by the same method.

(2) Transformation of the relationship between the amount of bound Triton X-100 and the concentration of remaining Triton X-100 into the relationship between the amount of bound Triton X-100 and the initial concentration of Triton X-100

Fig 4.15 gave the relationship between the amount of bound Triton X-100 and the concentration of remaining Triton X-100. However, in reality, a relationship between the amount of bound Triton X-100 and the initial concentration of Triton X-100 is preferred. The second relationship can be obtained from the first relationship by the following transformation:

$$K = C_b \times V_L / (N_c \times C_e) \quad (8)$$

Where: K is the slope of Fig 4.14, 10^{-5} mmol / 10^{10} cfu; C_b is the concentration of the bound Triton X-100, mmol; C_e is the concentration of the free Triton X-100, mmol; V_L is the volume of the liquid culture, L; and N_c is the amount of cells, cfu.

The initial amount of Triton X-100 is

$$C_0 \times V = C_b \times V + C_e \times V \quad (9)$$

Combine eq. 5 and eq. 6, then the amount of the bound Triton X-100 can be calculated by:

$$C_b \times V = K \times N_c \times C_0 / (1 + K \times N_c / V) \quad (10)$$

For the case of 0.6 OD_{600} in 100 mL liquid culture and a concentration of Triton X-100 at 0.2 CMC, the amount of bound Triton X-100 was

(3) Calculation of the surface coverage of anthracene by Triton X-100

The surface coverage of anthracene by Triton X-100 was roughly calculated as follows:

1. The hydrophobic tail, which is a branched 8 carbon unit (Fig 3.1), was assumed to bind to the anthracene particles. The sorption was assumed to be monolayer.
2. The particle size of anthracene was roughly visualized to be 0.7mm × 0.7mm × 0.1mm. Then the surface area of each particle was

   $2 \times (0.7\times10^{-3})^2 + 4 \times 0.7\times10^{-3} \times 0.1\times10^{-3} = 1.26\times10^{-6}\ m^2$
3. 120 particles weighed 3 mg.
4. The total surface area of 50 mg anthracene was

   $50/3 \times 120 \times 1.26\times10^{-6} = 2.52\times10^{-3}\ m^2$
5. The data of bond length are 1.53 Å, 1.09 Å for sp^3-sp^3 and sp^3-H respectively. Therefore, the dimension of the tail was approximated to be 6Å×3Å.
6. The total area of tails of 1 mmol Triton X-100 was

   $1\times10^{-3} \times 6.02\times10^{23} \times 6\times10^{-10} \times 3\times10^{-10} = 108\ m^2$
7. The area of tails of bound Triton X-100 in the growth condition was

   $1.56\times10^{-5} \times 108 = 1.68\times10^{-3}\ m^2$
8. The surface coverage of anthracene by Triton X-100 was

   $1.68\times10^{-3} / (2.52\times10^{-3}) = 67\%$

(4) Calculation of the surface coverage of the *Mycobacterium* sp. by Triton X-100

The surface coverage of *Mycobacterium* sp. by Triton X-100 was roughly calculated as follows:

1. The hydrophobic tail, which is a branched 8 carbon unit (Fig 3.1), was assumed to bind to the anthracene particles. The sorption was assumed to be monolayer.
2. 0.1 OD_{600} corresponded to 10^8 cfu/mL (Tongpim and Pickard, 1996)
3. The cell was rod-shaped and the size of a 3 week old cell varied from 0.5×1.2 μm to 0.4×2.8 μm (Tongpim, 1997). The average of the above two set of data was 0.5×2.0 μm. The cell was approximated as a cylinder, therefore, surface area of a single cell was

   $3.14 \times 0.5\times10^{-6} \times 2\times10^{-6} + 2 \times 3.14 \times (0.5\times10^{-6})^2/4 = 3.5\times10^{-12}$ m^2
4. The cell count represented by 0.6 OD_{600} for 100 ml liquid culture was

   $0.6/0.1 \times 10^8 \times 100 = 6\times10^{10}$ cfu
5. The total surface area of the cells was

   $6\times10^{10} \times 3.5\times10^{-12} = 0.21$ m^2
6. The total surface area of the bound Triton X-100 was

   $7\times10^{-4} \times 108 = 0.076$ m^2
7. Therefore, the surface coverage of *Mycobacterium* sp. by Triton X-100 was

   $0.076 / 0.21 = 36\%$

(5) Calculation of the effective surface area of cells for adhesion

The effective surface area for the *Mycobacterium* sp. to adhere to the anthracene particles was calculated as follows:

1. The effective area for cells to adhere to anthracene was considered to be its interception area. Therefore the effective area for a single cell was

   $0.5\times10^{-6} \times 2\times10^{-6} = 10^{-12}\ m^2$

2. The cell count for the initial inoculum was

   $0.07/0.1 \times 10^{8}$ cfu/ml × 100 ml = 7×10^{9} cfu

3. The total surface area of the cells was

   $7\times10^{9} \times 10^{-12} = 7\times10^{-3}\ m^2$

Appendix 5 Error Bar of the Slope of the Linear Regression Line

The error bar of the slope of the regression line of the control in Fig 4.6 will be calculated as an example. Error bars show the 95% confidence interval on the calculated growth rate, from the data of Fig 4.6.

The regression line is

$y = 0.0315\,x + 0.1626$

The variance of the slope is given by

Since

then the error bar of the slope is